Preparing for an influenza pandemic: Re-evaluating the use of the whole inactivated virus vaccine formulation

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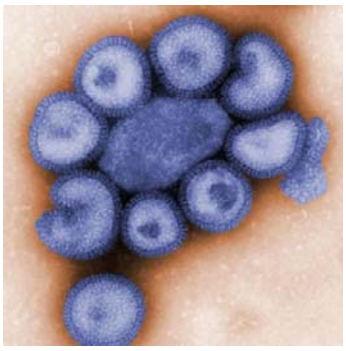
–A comparison of the immune responses after intramuscular or intranasal immunization with an influenza H5N1 whole inactivated virus vaccine in a murine model.



Monica Trondsen

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University of Bergen
Department of Biomedicine
and
Influenza Centre, The Gade Institute
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Front page illustration: The influenza A H1N1 virus.
Source: http://www.smh.com.au/world/how-the-flu-virus-works-20090430-anmp.html

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Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
AEC	9-amino 3-ethyl carbazole
APC	Antigen presenting cell(s)
ASC	Antibody secreting cell(s)
BALB/c	Bagg albino (inbred mouse strain)
BM	Bone marrow
CD	Cluster determinant
CHMP	The Committee for Medicinal Products for Human Use
ConA	Concanavalin A
CTL	Cytotoxic T lymphocyte(s)
DC	Dendritic cell
DMF	Dimethylformamide
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme linked immunospot assay
ER	Endoplasmic reticulum
FBS	Foetal bovine serum
FCS	Foetal calf serum
g	Relative centrifugal force
GMT	Geometric mean titre
HA	Haemagglutinin
HAU	Haemagglutinin units
HI	Haemgglutination inhibition
HIV	Human immunodeficient virus
HPAI	Highly pathogenic avian influenza
IFN	Interferon
Ig	Immunoglobulin
IM	Intramuscular
IN	Intranasal
IL	Interleukin
LM	Lymphocyte medium
LPAI	Low pathogenic avian influenza
M1	Matrix protein
M2	Ion channel protein
M-cell	Microfold cell
MALT	Mucosal-associated lymphoid tissue
MDCK	Madin-Darby Canine Kidney Cells
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NA	Neuraminidase
NALT	Nasal-associated lymphoid tissue
NCS	Newborn calf serum
NK	Natural killer cell
NP	Nucleoprotein
NS	Non-structural protein
OD	Optical density
OPD	o-Phenylenediamine
PA	Polymerase protein acidic

PAMP	Pattern-associated molecular pattern
PB	Polymerase protein basic
PBS	Phosphate buffered saline
PBS/T	Phosphate buffered saline/Tween
PPR	Pattern recognition receptor
PR8	A/Puerto Rico/8/34 (H1N1)
RDE	Receptor destroying enzyme
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
rpm	Revolutions per minute
SEM	Standard error of the mean
S-IgA	Secretory immunoglobulin A
ssRNA	Single stranded ribonucleic acid
SU	Subunit
Tc	Cytotoxic T cell(s)
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper cell
Thp	T helper precursor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
WHO	World Health Organization
WIV	Whole inactivated virus

Abstract

Influenza is a highly contagious respiratory disease which infects millions of people every year around the world. Influenza viruses undergo continuous antigen change and annual vaccination is an effective prophylactic measurer to combat the disease and to reduce morbidity and mortality. Recently, viruses of avian (and swine) origin have crossed the species barrier and caused infection in man, thus highlighting the need of vaccines that induce a strong and rapid immune response in a naïve population. Inactivated intranasally administered vaccines are not currently licensed, but would provide an attractive alternative to conventional intramuscular vaccination as they have the advantage of being non-invasive, easily administered and also induces a mucosal immune response.

Studies have shown that vaccines containing avian subtypes are poorly immunogenic and have lead to a re-evaluation of the use of whole virus vaccines which are more immunogenic than standard subunit vaccines and can be dose sparing. In the current study we have investigated the immune response after one or two doses of intramuscular or intranasal inactivated influenza H5N1 vaccine in BALB/c mice using whole virus vaccine containing 7.5 μg HA. Serum samples were analyzed using ELISA and HI assay, whereas lymphocytes from spleens and bone marrows were used to measure the number of antibody secreting cells (ASC) using ELISPOT. Splenic lymphocytes were stimulated *in vitro* and cytokine profiles were measured by multiplex bead assay. Additionally, we also measured local IgA from nasal washings by ELISA.

The intramuscular vaccine route induced a strong, long lasting humoral immune response and was characterized by a Th-1 cytokine profile after one vaccine dose, and a more mixed Th-1/Th-2 profile after two doses. In contrast, the intranasal vaccine route induced a strong local immune response after two vaccine doses and a good humoral immune response as well. This group showed a dominant a Th-2 profile, but also secreted cytokines associated with a Th-1 profile (IFN-γ and IL-2) and high levels of IL-17 (Th-17). Although both vaccine routes induced high levels of serum and HI antibody, the intranasally delivered vaccine also induced local IgA which provides the first line of defence against respiratory diseases. In addition, it would be suitable for use in developing countries as it is needle-free and easy to administer. We therefore suggest that an intranasally administered whole virus vaccine would be a good candidate for further investigation of a pandemic H5N1 candidate vaccine.

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1 Introduction

1.1 The influenza virus

Influenza remains one of the most common respiratory diseases in humans causing almost annual outbreaks. The viruses originate and circulate in east and Southeast Asia, and spread to Oceania, North America and Europe and finally to South America[1]. Influenza epidemics have been recorded since the 16th century[2], and it is still a phenomenon occurring during late autumn and winter in temperate climatic zones (e.g. Norway). Occasionally, at unpredictable intervals, pandemics occur and lead to high numbers of deaths round the world. The changing nature of influenza viruses and recently reported cases of viruses crossing the species barrier (e.g. H5N1, H7N7 and H1N1) has led to intensive research to develop new pandemic vaccines.

1.1.1 Taxonomy, structure and nomenclature

The influenza A virus is pleomorphic, either spherical or filamentous, ranging in diameter from 80-120 nm. It is an enveloped virus belonging to the family *Orthomyxoviridae* and is characterized by a negative-stranded RNA segmented genome. There are three known types of influenza viruses, Influenza A, B and C, which are characterized by antigenic differences in the two major structural proteins, the matrix protein (M) and the nucleoprotein (NP)[3]. Nucleoproteins encapsulate the genome's eight segments forming a ribonucleoprotein structure (RNP). These segments each code for one or two proteins, including the two important surface glycoproteins, Haemagglutinin (HA) and Neuraminidase (NA), which protrude as spikes from the viral envelope (Fig. 1.1). The function of HA is binding to the host cell and then enable fusion between the host cell endosomal membrane and viral membrane allowing release of viral nucleocapsids into cytoplasm of the host cell. HA is the host's main target antigen to which the protective antibody response is directed, and is the most abundant protein on the virus surface. The NA contributes to the release of newly produced virions by enzymatic cleavage of sialic acids. Additionally, NA has also been shown to play a key role in early infection probably by removal of decoy receptors which could inhibit virus binding to functional receptors[4].

The different influenza A viruses can generally be separated by the subtype of the surface proteins, where 16 subtypes of HA and 9 subtypes NA have been identified. As for the influenza types B and C, humans are the main host, whereas influenza A virus is known to have several animal hosts, including pigs, horses and birds, in addition to humans.

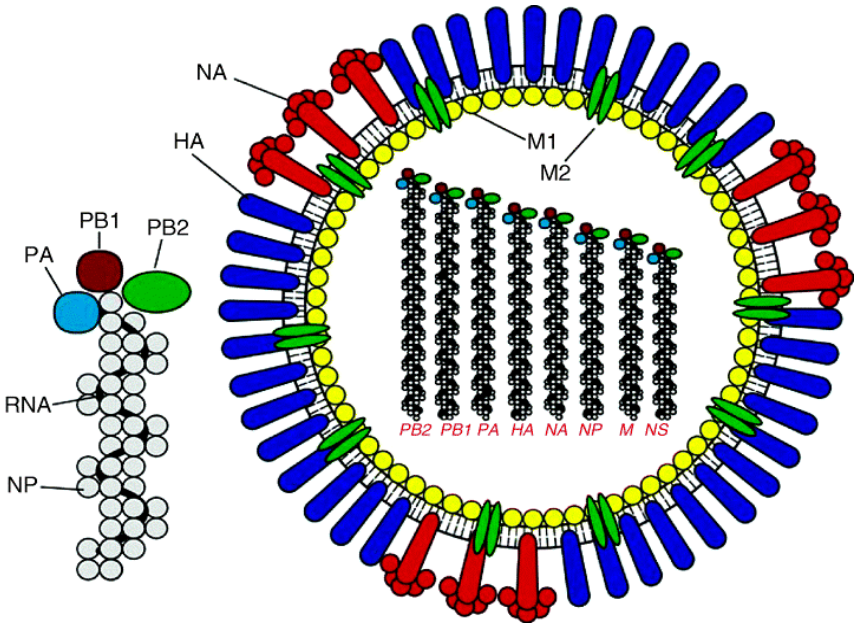


Figure 1.1. The structure of influenza virus. The illustration shows the virion with its two spike-like surface antigens HA (blue) and NA (red) and its segmented genome. The other viral proteins shown are; M1 (matrix protein), M2 (ion channel protein), PB2, PB1 and PA (polymerase subunits), NP (nucleoprotein) and NS (non-structural protein). On the left; the ribonucleoprotein complex. The figure is kindly provided by Karl A. Brokstad.

A standard nomenclature defined by the World Health Organization (WHO) is used to discriminate between the different types and strains of viruses. This naming for human isolates includes; type of virus, site of isolation, number of isolate and year of isolate, and for influenza A virus the subtype, e.g. A/Vietnam/1194/2004 (H5N1). If the virus is isolated from a species other than man, for instance a duck, this species will also be included (A/duck/Tuva/01/06 (H5N1)[5].

1.1.2 Replication cycle

The haemagglutinin of the virion initiates infection by binding to sialic acid containing receptors on epithelial cells in the upper respiratory tract. The binding induces endocytosis

of the virus into endocytic vesicles and then endosomes. In the endosome, the low pH triggers a conformational change in the HA exposing a fusion peptide that results in fusion of the viral envelope with the endosomal membrane. The M2 protein modulates the pH by forming a proton channel in the viral envelope and pumps in H^+ from the endosome into the virion. This results in uncoating and opening of a pore through which the viral RNPs are released into the cytoplasm and further transported to the nucleus. Unlike other (-) RNA viruses the influenza virus transcription and replication occurs in the nucleus of the host cell (Fig. 1.2).

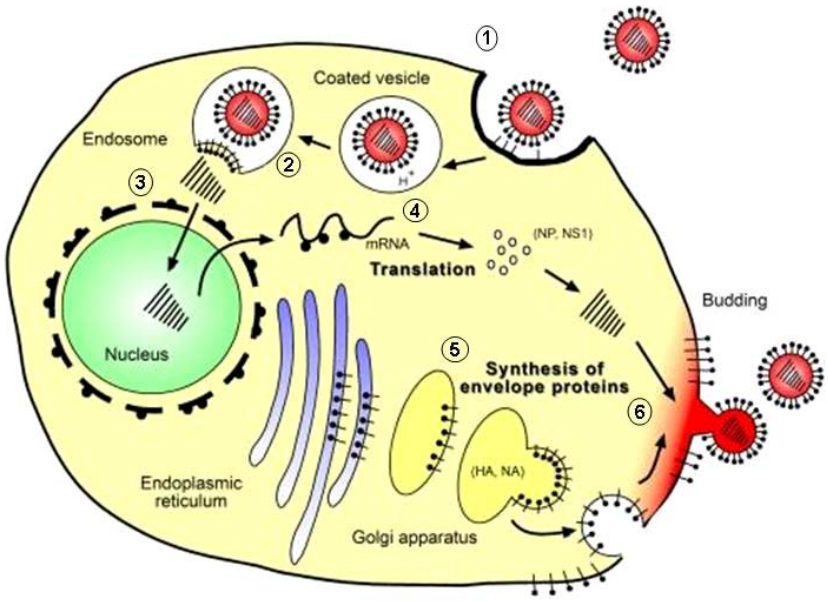


Figure 1.2. The replication cycle of influenza. 1) binding to host cell 2) uncoating and RNP release 3) transcription 4) translation on ribosomes 5) synthesis of new viral envelope proteins 6) assembly and budding of new progeny. See text for more details. The figure is modified from[6].

The mRNA is translated into new proteins in the cytoplasm and the gene segments produced in the nucleus associates with the nucleoproteins and are transported to the cell membrane. The HA and NA glycoproteins are modified in the endoplasmic reticulum (ER) and Golgi apparatus before transported to the cell surface. Finally, the proteins assemble, a new virus envelope is derived from the host cell membrane upon budding and new virions are released. An infection cycle lasts for approximately 8 hours.

1.2 Epidemiology

1.2.1 Antigenic drift and antigenic shift

The lack of proof-reading among RNA polymerases contributes to replication errors, mutations like substitutions, deletions and insertions. For that reason influenza viruses are continuously changing and they undergo two main antigenic variations, namely antigenic drift and shift. Antigenic drift is characterized by an accumulation of point mutations in the virion's surface proteins HA and NA, and renders the host's antibodies less effective in neutralizing the virus. This is the reason for recurrent epidemics and results in an annual update of the seasonal vaccines. Antigenic shift on the other hand, applies only to influenza A and occurs when the HA (and NA) segment is replaced with a novel HA segment to which man is immunological naïve. The segmented nature of the genome provides the influenza virus an opportunity to undergo antigenic reassortment. This phenomenon can lead to a new virus if a cell is doubly-infected by two different viruses and can give rise to a pandemic strain.

Three pandemics occurred during the last century. The 1918-1919 "Spanish Flu" killed an estimated 20 to 50 million people worldwide, and was caused by a direct adaptation of a virus of avian origin (the H1N1 subtype) which transmitted efficiently in humans[7]. In 1957 the H1N1 type was replaced by a new subtype H2N2, believed to have been created by an intermediate host (pig) infected with both avian and human influenza, and claimed approximately 1-2 million lives worldwide. The 1968 pandemic "Hong Kong Flu", introduced a new shift to the H3N2 variant, which killed an estimated one million people worldwide. The WHO has now declared a new pandemic after a novel virus of the H1N1 subtype, swine origin influenza A (H1N1) ("Swine Flu") emerged in Mexico (March-April 2009)[8]. This virus contain a combination of gene segments which that have not previously been found in any species (swine nor humans)[9], and has up to date infected hundreds of thousands of people around the world.

The first human infection with H5N1 subtype occurred in Japan in 1997[10], and this subtype has since then been considered a pandemic threat. Fortunately, only limited person-to-person transmission has been seen[11].

1.2.2 Influenza A and ecology

Wild birds, particularly waterfowls and shorebirds are the main natural reservoir of all the influenza A viruses, and of the 16 subtypes of HA and 9 subtypes of NA known, all of them have been isolated from birds[12]. Only a few subtypes have established themselves in humans (HA [H1, H2 and H3] and NA [N1 and N2]), and other mammals such as pigs (HA [H1 and H3] and NA [N1 and N2]), horses (H3N8 and H7N7) and dogs (H3N8). However, there are in general strong barriers to interspecies transmission that prevent influenza virus adaptation to new hosts (reviewed in[13]).

In wild birds influenza is not a respiratory virus, but a less harmful intestinal infection which usually doesn't results in disease. Wild birds shed large quantities of virus in their faeces and if domesticated birds, including turkeys, ducks and chickens are infected, they may get seriously ill and die. Based on the severity of the disease they cause, the viruses infecting poultry can be divided in two groups. The most severe form is termed highly pathogenic avian influenza (HPAI), and has been recognized as a disease entity since 1878 (then called fowl plaque)[14]. The two subtypes causing HPAI are H5 and H7. HPAI can be serious for farmers which suffer huge economical losses as the mortality rate often is close to 100 %, and the disease is extremely contagious. The other form, low pathogenic avian influenza (LPAI) is recognized by milder symptoms like ruffled feathers, mild respiratory disease and a drop in egg production (reviewed in [15]). A big problem is that the virus can mutate from a LPAI form to a HPAI form, and thus cause serious outbreaks among poultry. These HPAI viruses can infect humans which are in close contact with infected poultry, for instance in countries like Indonesia, Vietnam and Egypt where the population lives in close proximity to their livestock.

A major determinant of the molecular basis of virulence lies in the HA protein. During replication, the HA (HA0) is cleaved by the host's proteases into HA1 and HA2 and this post-translational modification is essential for virus infectivity. LPAI only have one basic amino acid in the cleavage site[16], and thus cleavage can only occur by definite hosts proteases found in the respiratory tract and intestine. In contrast, HPAI contains multiple basic amino acids which allow cleavage by a wide range of proteases in other tissues. In this way, replication of the virus can occur throughout the birds vital organs and tissues, and can lead to a rapid death (reviewed in [13,15]).

1.2.3 Clinical features of influenza

Annually, influenza causes a great number of deaths, 250 000 to 500 000 worldwide[17] and approximately 1000 deaths in Norway[18]. All age groups are susceptible to infection. The disease has an average incubation time of 2 days (1-4 days), and is spread by aerosols produced upon sneezing and coughing. Symptoms are sudden onset of fever, runny nose, sore throat, headache, muscle pain (myalgia) and fatigue, and the illness lasts for 7-10 days with a normal recovery. A common complication is secondary bacterial pneumonia, which can be fatal for the elderly, immunodeficient and others with underlying chronic illnesses[19].

1.2.4 Human infection with H5N1 viruses

The human influenza viruses have a tissue tropism for binding terminal sialic acid linked to galactose by α-2,6 linkages on host cell receptors. Avian viruses, on the other hand, preferentially recognize α-2,3 linkages[20] which is common in avian gut tissue. These viruses were thought to have little affinity for human respiratory tissues[21] and therefore replication was restricted[22], however it has later been found that H5N1 viruses bind strongly to cells in the lower respiratory tract in humans[23]. Receptors for both avian and human influenza viruses are present in porcine epithelium and pigs have for that reason been considered a "mixing vessel" for reassortment of new viruses (reviewed in[13]). HPAI H5N1 virus transmission occurs directly from birds to humans, without an intermediate host.

The incubation time after exposure to sick poultry seems to be longer than for seasonal influenza and up to 8 days have been reported (average 2-5 days). Common symptoms are fever, diarrhoea, cough and shortness of breath, and pneumonia that generally appears to be of primary viral origin (reviewed in [13, 24]). This is usually complicated with acute respiratory distress syndrome, and in severe cases multiorgan failure. As in poultry, the virus may spread to tissues outside of the respiratory tract (e.g. lymph nodes and liver). Another feature is cytokine dysregulation, an intense inflammatory reaction to the high amounts of virus[25]. So far 436 persons have been infected and 262 persons died from H5N1 infection (July 2009)[26]

1.3 Prophylaxis and therapy

1.3.1 Vaccines

Vaccination is the most effective way of preventing influenza infection and to reduce morbidity and mortality. Every year nearly 300 million doses of influenza vaccine are produced intended for world wide immunization[27]. The vaccine is recommended for groups at high-risk of complications; individuals ≥ 65 years, small children, health care workers and persons with chronic medical conditions[17]. The disease has a large economic impact on society, due to hospitalization, visits to medical practisers and absenteeism. Vaccination is important for herd immunity as it hinders efficient spread of infection. The vaccine is 70-90 % effective in preventing illness amongst healthy adults, and reduces illness and complications by up to 60 % in elderly persons[17]. But most importantly, it reduces influenza associated deaths by 80 %[17].

Due to antigenic drift, immunity to seasonal influenza is not long lasting and the vaccines require annual updates. The seasonal influenza A H1N1 and H3N2 are the two subtypes currently circulating in the human population, and the trivalent seasonal vaccines therefore includes one strain from each of these two subtypes, in addition to influenza B. Both influenza A and B can give rise to epidemics, whereas type C only has a low pathogenicity in humans and is not included in the vaccine. The composition of the vaccine candidates is recommended by the WHO annually, based on scientific consensus. The WHO has a global influenza surveillance network where currently 128 national influenza centres are active [28], to allow for rapid vaccine strain selection.

1.3.2 Propagation of virus for vaccine production

Currently, the production of commercial vaccines occurs almost extensively in embryonated hens' eggs. The majority of all influenza strains will to some extent replicate in eggs and the enclosed environment does not require any aseptic conditions[29]. However, there have been problems with growing the H5N1 virus because of its virulence resulting in killing the embryo. The use of reverse-genetics technology has overcome this problem by engineering the original HA (without the basic amino acids at the cleavage site) and NA onto a standard influenza A vaccine strain[30]. A strain called PR8 (A/Puerto Rico/8/34 (H1N1)) has been used as a donor strain for the last decades to prepare "high-growth" influenza A reassortants for egg-based vaccine production[3]. In addition, vaccines produced by using mammalian cell lines (MDCK and Vero cells) have been licensed[3], and also the

use of plant cells are being investigated[31]. This could alter the dependence on eggs, and allow for rapid large scale production in case of a pandemic**.** Furthermore, people with egg allergies would benefit from for instance cell culture-grown vaccines, as they are not recommended for immunization with egg-grown vaccines[3].

1.3.3 Inactivated influenza vaccines

Inactivated influenza vaccines account for more than 90 % of the world's vaccine market[32]. The vaccines are made from purified egg grown virus and there are three main formulations; whole virus, split virus (also called subviron) and subunit (SU) vaccines (Fig. 1.3). Influenza viruses are normally inoculated into the allantonic cavity of the egg, and the allantonic fluid containing viruses is harvested[29]. First, the virions are concentrated by centrifugation and inactivated by formalin or β-propiolactone. In the whole virus vaccine formulation the viral particle is kept intact including the surface proteins and RNA segments. The split virus vaccine is prepared by disrupting the viral lipid envelope with detergents (e.g. Triton-X or deoxycholate), and the formulation contains all parts of the virus, although some proteins are removed to some extent. The third formulation, the subunit vaccine, is further purified and contains only highly purified surface antigens, haemagglutinin and neuraminidase[3].

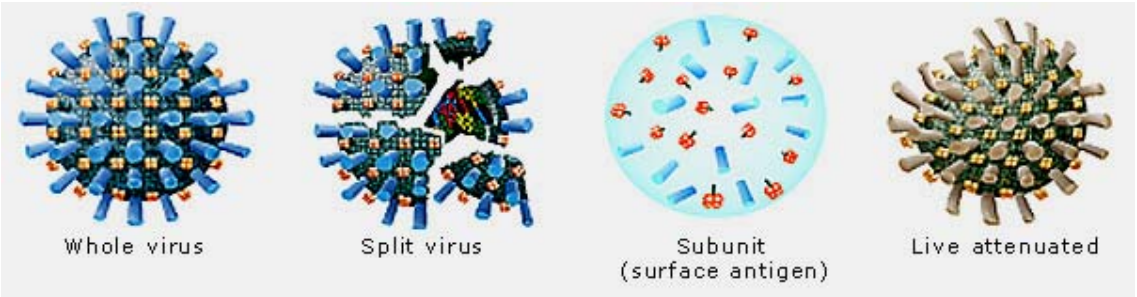


Figure 1.3. Influenza vaccine formulations. The figure depicts four different vaccine formulations; whole virus, sub unit subunit and live attenuated influenza vaccine (licensed for human use in the US and Russia, and are administered intranasally as a spray. The containing viruses are adapted to growth at low temperatures (25 ºC), and replicates poorly at higher temperatures (39 ºC)[33]). From reference [34].

The whole virus vaccine is highly immunogenic and is still used in some countries, but most vaccines produced after the 1970's have been split or subunit, because of increased reactogenicity observed using whole virus[35]. Split and SU vaccines are still immunogenic, and the dissolution of the viral envelope and removal of additional viral components are

associated with reduced reactogenicity[3]. Common local side reactions after vaccination are pain at the injection site, acute inflammatory reactions, erythema and induration. Systemic reactions such as fever, myalgia and headache may occur, but are much less frequent than local reactions, and have in recent trials not been commonly observed[3]. It is important that the vaccine induces minimal of side effects since the target groups often already have underlying medical conditions. Moreover, less side reactions makes vaccination more attractive.

1.3.4 Immunological adjuvants

Vaccines containing novel influenza subtypes have been shown to be weakly immunogenic[36], and to obtain the best possible immune response after vaccination most candidate pandemic vaccines have required immuno-stimulating substances called adjuvants. Adjuvants are intended to enhance the immune response to the vaccine antigens by stimulating receptors of the innate immune system; pathogen associated molecular patterns (PAMPS) and toll-like receptors, or enhance the action of dendritic cells (described below)[37].

The aluminium salts and the MF-59 (oil-in-water microfluidized emulsion) adjuvants are frequently used to enhance the immune response of pandemic candidate vaccines administered parenterally[38](reviewed in[39]). The use of adjuvants can be dose-sparing, as lower antigen doses are needed to elicit a satisfying result. Mucosal adjuvants like *E.coli* labile-toxin are used in many studies, but are not licensed for human use due to side effects[40]. However, other bacteria-derived promising mucosal adjuvants are currently tested out[41, 42].

1.3.5 Antiviral drugs

There are two classes of antiviral drugs approved for human use, the adamantanes and the neuraminidase inhibitors. Only the latter is licensed in Norway. The adamantanes, Amantadine and Rimantadine are effective against for influenza A virus and their main target is the transmembrane domain of the M2 ion-channel protein. This treatment will prevent uncoating and release of RNPs. However, a widespread resistance to these drugs among influenza viruses is seen and they are currently not recommended in the US[3]. The main target of the NA inhibitors Zanamivir (Relenza) and Oseltamivir (Tamiflu) is the

active enzymatic site of neuraminidase. Treatment with NA inhibitors will cause aggregation of viruses at the cell surface and prevent effective release of new virus. The two drugs have activity against both influenza A and B[3]. Oseltamivir resistance is widespread among circulating seasonal influenza A (H1N1)[43,44], and also some strains of H5N1 have shown resistance[45].

1.4 The immune response to influenza

The immune system is a highly complex system of different cells and molecules that protects the body from invading pathogens. It can be differentiated into two arms, innate (natural) immunity and adaptive (specific/acquired) immunity, where a fundamental difference between the two is immunological memory upon re-encounter with a pathogen. Whereas the innate immune system deals with all intruders in the same way and lacks specific memory, the adaptive immune system can recognize and generate memory, and thus act rapidly upon re-encountering with the same pathogen. These two arms act closely together and innate immunity may keep the infection in check before adaptive immune responses are developed.

1.4.1 Innate immunity

The innate immune system provides the initial defence against a pathogen and consists of cellular and biochemical defence mechanisms. Its components include the skin, mucosal epithelia and antimicrobial chemicals such as defensins and cathelicidins. Since influenza is a respiratory virus and enters through the respiratory tract, mucosal immunity is important in preventing infection.

The cells of the innate system, macrophages and neutrophils, phagocytize and kill microbes along with natural killer (NK) cells which induce lysis of virus infected cells. The two former cells, in addition to endothelial and dendritic cells (DCs), express pattern recognition receptors (PRRs) which are specific for structures shared by groups of microbes identified as PAMPs (pathogen-associated molecular patterns). Interaction with microbial products can transmit signals that lead to activation and transcription of cytokine genes. An example is toll-like receptor 7, which is activated by influenza ssRNA and participates in viral clearance by inducing secretion of type 1 interferons (IFNs) by

plasmacytoid DCs[46]. Fever is also a part of the innate systems defence, and is induced by Interleukin-1 (IL-1), IL-6 and tumour necrosis factor-α (TNF-α) secreted by macrophages. In addition, these cytokines also activates NK-cells (reviewed in [47]).

Communication between the innate and adaptive system is carried out by professional antigen presenting cells (APCs). Immature DCs which reside in most peripheral tissue are considered the most potent antigen presenting cells. Here, they capture foreign products and travel to lymph nodes where they present antigens to naïve T lymphocytes.

1.4.2 Adaptive immunity

The main components of the adaptive immune system are the lymphocytes and their secreted products; antibodies and cytokines. The adaptive immune system can be divided into a humoral arm which involves B-cells and antibody production and a cell-mediated arm where T-cells kill infected cells or stimulate other cells by production of cytokines (Fig. 1.4).

1.4.2.1 Cell mediated immune responses

Precursor T-cells migrate from the bone marrow to the thymus and mature in the absence of antigens. Thereafter, naïve T-cells continuously re-circulate to the lymph nodes, searching for antigens fitting their unique T-cell receptor (TCR). Cell mediated responses are performed by two types of T-cells, $CD8^+$ and $CD4^+$ cells. Antigens taken up by DCs (APCs) are processed and presented on the cell surface by MHC molecules. $CD4^+$T-cells recognize peptides on MHC class II (described below), whereas $CD8^+$T-cells recognize and bind to MHC class I molecules. Upon this binding the $CD8^+$T-cells become activated leading to differentiation into cytotoxic T lymphocytes (CTL), clonal expansion and migration to the site of infection. CTLs are essential in the clearance of primary viral infection, and appears early in the immune response. Infected cells are destroyed by the release of cytolytic perforin and granzyme B from granules in CTL, or through Fas-FasL interactions leading to cell lysis and apoptosis (Fig. 1.4) (reviewed in[47, 48]). Furthermore, the CTL response to influenza A is broadly cross-reactive between virus strains because the NP and M proteins are conserved between strains and is important in the recovery from primary infections[49], and influenza pneumonia[50].

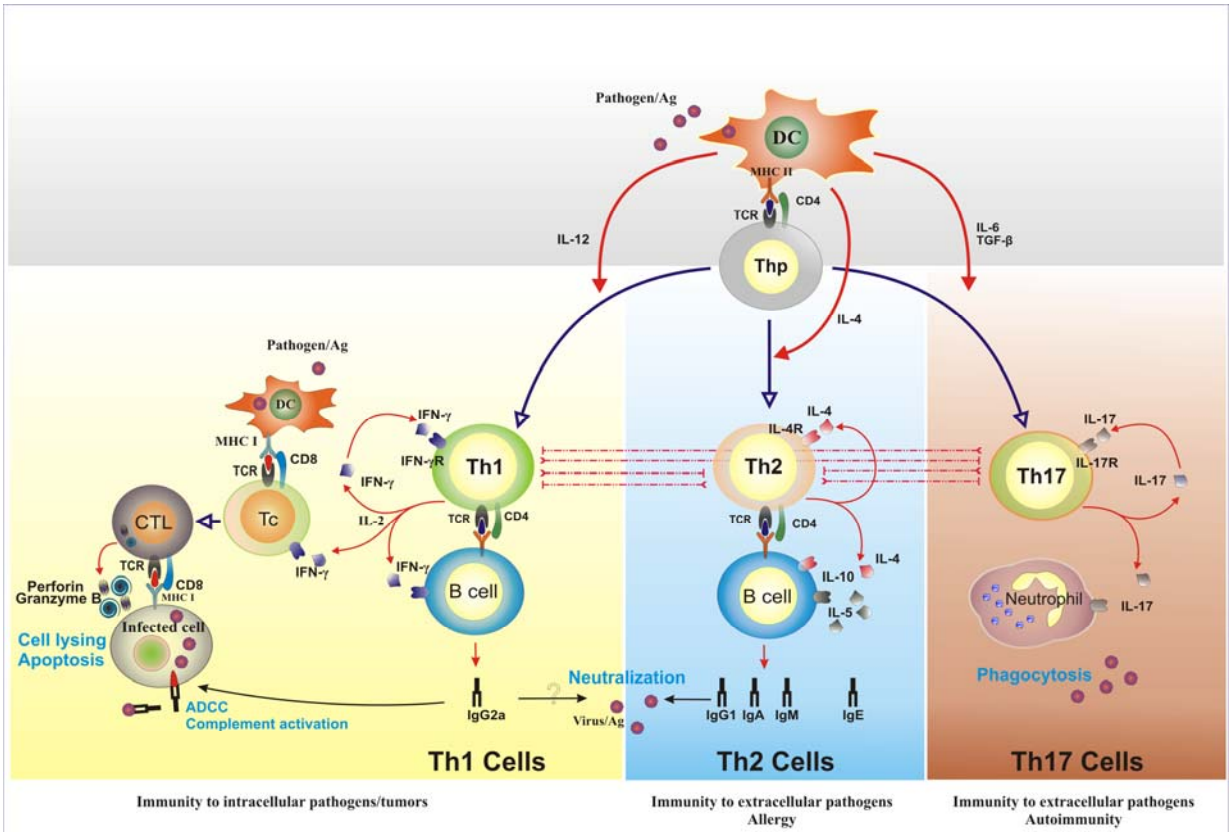


Figure 1.4. An illustration of the adaptive immune responses induced following an infection or vaccination. The three different T-helper subsets of CD4$^+$ cells are shown (Th1/Th2 dichotomy and Th-17). Details are described in the text. The abbreviations used: Ag: antigen, DC: dendritic cell MHC: major histocompatibility complex, CD: cytoplasmic domain, Tc: cytotoxic t-cell, TCR: t-cell receptor, IL: interleukin, IFN: interferon, TGF: transforming growth factor, CTL: cytotoxic t-lymphocyte, ADCC: antibody-dependent cell-mediated cytotoxicity, APC: antigen presenting cell, Th: t-helper, Thp: t-helper cell precursor, R: receptor, Ig: immunoglobulin. The figure is kindly provided by Abdullah Madhun.

1.4.2.2 T-helper cells and cytokines

Helper T-cells (CD4$^+$) play a central role in the regulation of the different components of the immune response. Following contact with DCs the T-cells differentiate into functionally distinctive subsets of effector T-helper cells (Th). Particular cytokines secreted by the DC influences this decision, for instance IL-12 stimulates a Th-1 profile (Fig. 1.4). Th-1 cells mainly secrete IFN-γ, IL-2 and TNF-β. On the other hand, DC-derived IL-4 promotes the development of the Th-2 subset which secrete IL-4, IL-5, IL-6, IL-10 and IL-13[51]. Some T-cells become regulatory T-cells, whose functions are maintaining peripheral self-tolerance and immune suppression (reviewed in[52]). The Th-1/Th-2 dichotomy was described in 1986 by Mosmann and colleagues[51]. The two subsets

are mutually inhibitory; IFN-γ secreted by Th-1 cells suppresses Th-2 proliferation and IL-4 secreted by Th-2 cells suppresses proliferation of Th-1 cells[53, 54] (Fig. 1.4). In mice, a Th-1 profile enhances a cytotoxic immune response and production of IgG2a antibodies, whereas to a Th-2 profile stimulates B-cells and production of antigen specific IgG1, IgA and IgM antibodies.

Some years ago, a third subset of $CD4^+$ T-cells producing IL-17 was described, termed T-helper-17. Th-17 cells develop in response to IL-6 and TGF-β (in mice) and secrete IL-17, which stimulates neutrophils and phagocytosis of extracellular pathogens (Fig. 1.4). However, the Th-17 subset may also promote tissue inflammation and autoimmunity [55, 56].

1.4.2.3 The humoral immune response

B-cells are produced in the bone marrow (BM) and after maturation they circulate and reside mainly in secondary lymphoid tissues such as spleen, lymph nodes and mucosa-associated lymphoid tissue (MALT). After encounter with an antigen, and additional help from Th-cells, they differentiate into effector B-cells which secrete antigen-specific antibodies (plasma cells), or memory B-cells. Some of these cells are long-lived plasma cells which after a viral infection migrate to the BM and secrete antibodies over long periods of time[57].

There are five classes of antibodies; IgM, IgD IgG, IgA, and IgE, which are small glycoproteins composed of two heavy chains and two smaller light chains, which exist as monomers, dimers and pentamers. This structure enables for instance IgM, which is a pentamer, to bind five antigens at the same time. Mature B-cells co-express membrane bound IgD and IgM on their surface, and IgM are the first to appear in a primary immune response to an infection (Fig. 1.5). At later stage of the immune response, they undergo a process called affinity maturation, where the B-cell clones with the highest affinity for the antigen are selected and antibody class switching from IgM to IgG, IgA or IgE. Class switching by somatic hypermutation is controlled by cytokines secreted by Th-cells, and occurs in germinal centres (areas in e.g. spleen) (reviewed in[58]). IgG dominates in serum, whilst IgA is the dominant antibody class in mucosal epithelia (described below). IgE is an important mediator in hypersensitivity reactions such as allergy. The role of IgD is more unclear. Antibodies have three major functions; they neutralize antigen, coat microbes (opsonization) and target them for phagocytosis by macrophages and neutrophils.

Moreover, they activate complement proteins which also enhance phagocytosis. Antibody-dependent cell-mediated cytotoxicity (ADCC) is also induced by antibodies (IgG2a in mice) (Fig. 1.4) where NK-cells are targeted to infected Ig-coated cells for lysis.

In mice there are four subclasses of IgG; IgG1, IgG2a, IgG2b and IgG3. IgG1 can be used as a Th-2 marker, and is associated with a predominant humoral response. IgG2a, in contrast, can be used as a Th-1 marker and is associated with a cellular response (ADCC and $CD8^+$ T-cell cytotoxicity). After vaccination the concentration of IgG2a correlates with clearance of virus and an increased protection against infection, whereas IgG1 more effectively neutralize virus[59]. Thus, both subclasses play an important role in combating an influenza infection.

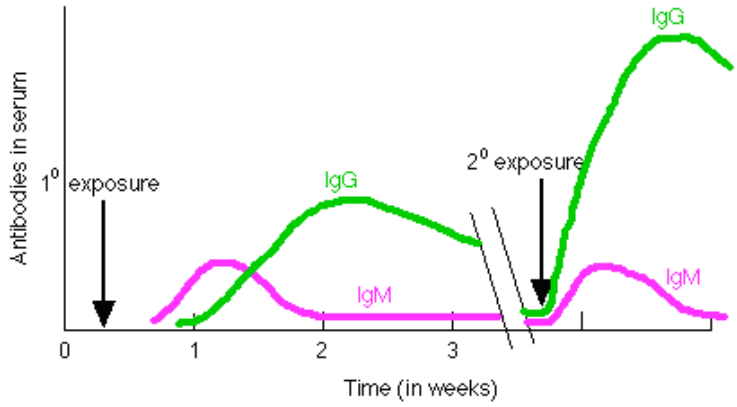


Figure 1.5. Primary and secondary immune responses after exposure to an antigen. The figure is from reference[60].

A second exposure to the same antigen induces a more rapid and vigorous secondary immune response (Fig. 1.5). The secondary response is very effective since memory cells quickly recognize the antigen. In addition, the response is long-lived and dominated by high-affinity serum antibodies, especially IgG. In influenza infection IgG transudes over the alveolar walls in the lungs, and may prevent illness and infection[61]. Therefore, an influenza vaccine should induce high levels of this antibody class.

1.4.2.4 Mucosal immunity

The first line of defence against influenza is the mucosa of the nasal cavity and the respiratory tract which is lined with ciliated epithelium and a layer of mucus. The nasal mucosa is particularly rich in dendritic cells. They receive exogenous antigens sampled at

the mucosal surface by specialized cells called microfold-cells (M-cells) (Fig. 1.6). In mice, the mucosal immune response is induced in the nasal-associated lymphoid tissue (NALT), which consists of paired lymphoid organs containing high numbers of T- and B-cells[62].

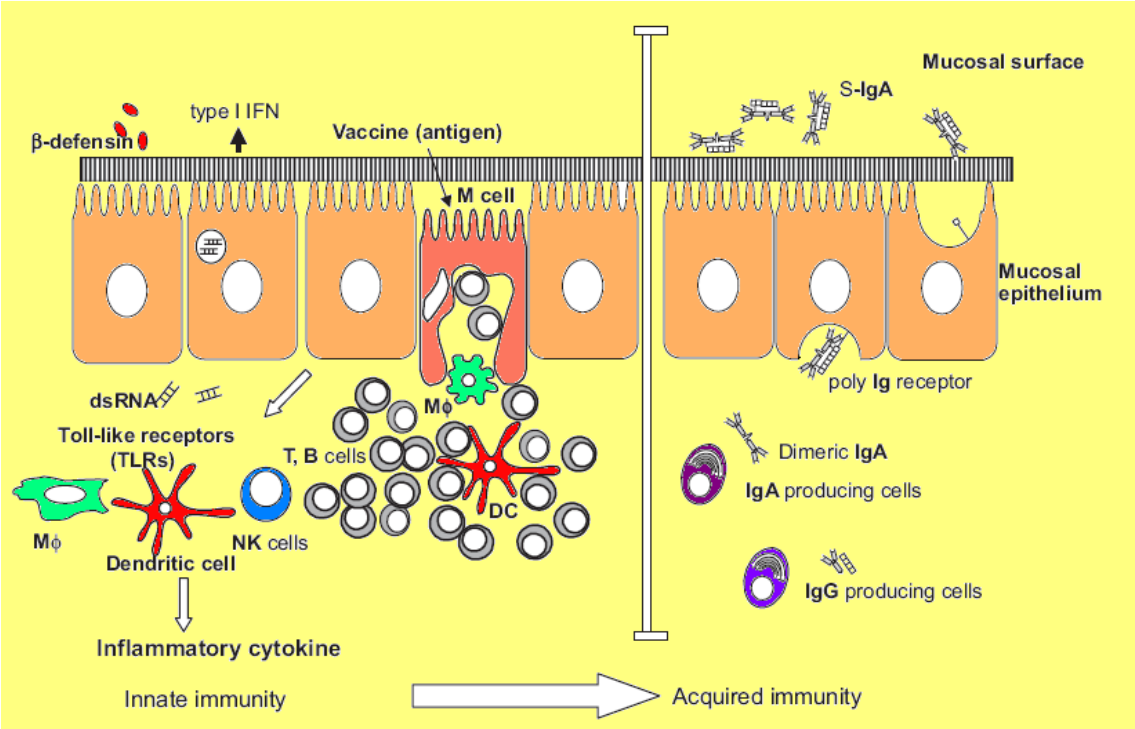


Fig 1.6. Innate and adaptive mucosal defence mechanisms. The picture illustrates cells and molecules involved in the immune response after vaccination or infection. See text for details. The abbreviations used: IFN: interferon, Mϕ = macrophage, NK-cells: natural killer cells, DC: dendritic cell, S-IgA: secretory IgA, M cell: microfold cell. Details are described in the text. Adapted from Hasegawa *et.al* 2009 (reference[63]).

Following activation of B-cells, IL-10 or TGF-β secreted by Th-cells induce them to become IgA secreting plasma cells (reviewed in [64]). The secreted IgA reach the effector site by trans-epithelial transport. The dimeric IgA (D-IgA) binds to a polymeric IgA receptor localized on epithelial cells and is transported in a vesicle through the cell to the mucus lining the respiratory tract. At the surface the receptor is cleaved by specific proteases and IgA is released as secretory IgA (S-IgA) (reviewed in[47,64]). During this transport, the D-IgA can also bind and neutralize newly synthesized viral proteins in virus-infected cells and thereby prevent spread of new progeny.

S-IgA antibodies are highly effective in preventing infection in the upper respiratory tract. Here, S-IgA reduces viral shedding, effectively binds and neutralizes viruses, and also

protects against drifted influenza strains. Their action on the mucosal surface enables them to bind to and neutralize the virus even before it has infected an epithelial cell. As mucosal immunity plays such a major role in the defence against influenza infection, intranasal vaccines stimulating the immune system in the respiratory tract mucosa are an attractive candidate pandemic vaccine.

1.5 Aims of the study

The intramuscular or deep subcutaneous administration route is currently preferred for inactivated influenza vaccines[3]. In contrast, no inactivated intranasally administered vaccines are licensed, although this route is non-invasive and needle-free. Since conventional influenza vaccines are split and subunit vaccines, the use of whole inactivated virus vaccine (WIV) which is more immunogenic, is an interesting formulation for studying as a pandemic candidate vaccine.

Influenza A H5N1 is considered a pandemic threat and many vaccine studies have been conducted to date. A recent study showed that WIV vaccine using a dose of 7.5 µg HA without adjuvant gave a satisfying immune response in man[65]. However, it was only tested parenterally.

The aim of this thesis was thus to investigate the differences of the immunological profiles induced in mice after intramuscular and intranasal vaccination using the WIV vaccine formulation, an antigen dose of 7.5 µg HA and the pandemic influenza A H5N1 candidate vaccine.

2 Materials

2.1 Mice

Name	Supplier
BALB/c – 6-8 weeks old, albino, female	Taconic M&B A/S, Denmark

2.2 Vaccines, viruses and anaesthetics

NIBRG-14 Influenza whole virus H5N1 (A/Vietnam/1194/2004) 1040150-0003. Beta-propiolactone inactivated virus concentrate (1078 µg HA/ml) (Coating antigen - ELISPOT, ELISA)	Archimedes Development Ltd. Albert Einstein Centre, United Kingdom
Influenza H5 virosomal vaccine, NIBRG-14 (Lymphocyte activation medium)	Crucell B.V. Holland
Whole virus vaccine, 182.1 µg HA/mL (A/Vietnam/1194/2004), H5N1, NIBRG-14 (Vaccination, HI-assay)	Crucell B.V. Holland
Anaesthetics:	
Rompun® Vet (Xylazine) (20 mg/ml)	Bayer, Germany
Ketalar (Ketamine) (50 mg/ml)	Pfizer, USA
Euthanasia:	
CO_2-chamber	Scanbur A/S, Denmark

2.3 Blood

Turkey red blood cells (10 %) in PBS	National Institute for Biological Standards and Control (NIBSC), United Kingdom

2.4 Antibodies

Name	Catalogue number	Supplier
Capture antibody (1 mg/mL):		SouthernBiotech, USA
Goat anti-mouse IgA	1040-01	
Goat anti-mouse IgG	1030-01	
Goat anti-mouse IgG1	1070-01	
Goat anti-mouse IgG2a	1080-01	
Goat anti-mouse IgM	1020-01	
Immunoglobulin standards		
Mouse IgA (1 mg/mL)	M1421	Sigma, USA
Mouse IgG (1 mg/mL)	15381	Sigma, USA
Mouse IgG2a (1 mg/mL)	M9144	Sigma, USA
Mouse IgG1 (0.5 mg/mL)	0102-14	SouthernBiotech, USA
Biotinylated antibodies (Goat anti-mouse)		
		SouthernBiotech, USA
IgA (0.5mg/mL)	1040-08	
IgG (0.5mg/mL)	1030-08	
IgG1 (0.5 mg/mL)	1070-08	
IgG2a (0.5mg/mL)	1080-08	
IgM (0.5mg/mL)	1020-08	

2.5 Reagents and chemicals

Name	Cat. No	Supplier
AEC (9-amino 3-ethyl carbazole) (20 mg tablets)	A-6926	Sigma, USA
Bovine Serum Albumin (BSA)	A-6793	Sigma, USA
Citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$)	1.00244	Merck, Germany
Dimethylformamide (DMF)	10322	BHD AnalaR, England
Di-sodium hydrogen phosphate anhydrous (Na_2HPO_4)	1.06586	Merck, Germany

Extravidin Peroxidase (Extravidin PO)	E-2886	Sigma, USA
Counting beads: Negative Control (FBS) Compensation Particles Set	552843	BD Biosciences, USA
Foetal bovine serum (FBS)	14-701F	BioWhittaker, Belgium
Glacial acetic acid ($C_2H_4O_2$)	1-06268	Merck, Germany
Hepes buffer (1M)	H0887	Sigma, USA
Hydrogen peroxide, H_2O_2, 30 %	H1009	Sigma USA
Ionomycin	10634	Sigma, USA
Lymphoprep ™	1114545	Axis-Shield PoC A/S, Norway
Minimal Essential Medium (MEM) nonessential amino acids (100x)	11140-035	GIBCO, UK
Mercaptoetanol (2-ME)	M-7522	Sigma, USA
Newborn Calf Serum (NCS)	ECS0070L	Euroclone, Italy
Ortho-phenylediamine dihydrochloride (OPD, 10 mg)	P-8287	Sigma, USA
Penicillin/Streptomycin/Fungizone (PSF)	17-745E	BioWhittaker, Belgium
Phorbol myristate acetate (PMA)	P8139	Sigma, USA
Potassium di-hydrogen phosphate (KH_2PO_4)	1.04847	Merck, Germany
Receptor destroying enzyme (RDE)	340122	Denka Seiken CO, Japan
RPMI (Roosewell Park Memorial Institute) medium	21875-034	GIBCO, UK
Sodium acetate trihydrate (CH_3COONa)	1-06267	Merck, Germany
Sodium chloride (NaCl)	1.06404	Merck, Germany
Sodium pyruvate (100 mM)	S8636	Sigma, USA
Sulphuric acid 18.4 M (H_2SO_4)	112080	Merck, Germany
Trypan blue stain (0.4 %)	17-942E	BioWhittaker, Belgium
Tween 20: Polyoxylene-Sorbitan monolaurate	P-1379	Sigma, USA

2.6 Kits

Mouse Cytokine Grp I X-Plex Assay (Cytokine 6-plex - IL-2, IL-4, IL-5, IL-10, IL17, IFN-g) Cat. No X6000006RJ		Bio-Rad Laboratories, USA
Bio-plex Reagent Kit	(171-304000)	
Bio-plex Calibration Kit	(171-203060)	

2.7 Plates/equipment

Name		
Elisa plates, F-bottom	(655001)	Greiner, Germany
Elispot - Multiscreen®, HA	(MAHA N45 50)	Millipore, UK
Multiplex - Multiscreen®, HTS™, BV	(MSBVN1250)	Millipore, UK
HI – V96 MicroWell™ Plates	(249570)	Nunc Brand Products, DK
Nunc Immuno™ Wash 12 Plate washer		Nunc Brand Products, DK
Microvette® CB 300-		
System for capillary blood collection	(16.440.100)	Sarstedt, Germany

2.8 Instruments

Name	Supplier
Labsystems (Original) Multiscan MS	Labsystems
Immunoscan ™ Elispot reader	C.T.L Europe GmbH, Germany
Bio-Plex ™ 200 System Powered by Luminex XMAP ™ Technology	Bio-Rad Laboratories, USA
BD FACS Canto ™ Flow Cytometer (No.337175)	BD Biosciences, USA
Forma Scientific" bio-freezer Forma 8438 (-80°C)	LabTrader
Knf Lab Laboport vacuum pump	Bio-Rad Laboratories, USA
Heidolph Titramax 100 Vibrating platform shaker	Heidolph Instruments, Germany
Centrifuges:	
Heraeus Labofuge 400R – FunctionLine	Thermo Scientific
Spectrafuge mini, 240V (no. C 1301)	Labnet International, Inc.
Eppendorf Centrifuge 5424, 230V (no. 0006928)	Eppendorf International

2.9 Software

Ascent Software Version 2.6 (ELISA)	Labsystems
Program BioPlex manager 5 (Multiplex)	Bio-Rad, USA
ImmunoSpot 4.0 Academic and Immunoscan Professional (ELISPOT)	C.T.L, Europe, Germany

2.10 Solutions, buffers, medium

B-cell medium, 100 mL

- 86 mL RPMI 1640 medium supplemented L-glutamine
- 1 mL 0.1 mM nonessential aminoacids
- 1 mL 10 mM Hepes pH 7.4
- 1 mL 1mM sodium pyruvate
- 1 mL PSF
- 100 µL $5x^{10-5}$ M 2-ME
- 10 mL heat-inactivated FBS

PBS/NCS (20 %) 250 mL

- 50 mL NCS (newborn calf serum) to 200 mL sterile PBS

PBS/FBS (5 %) 250 mL

- 12.5 mL FBS (foetal bovine serum) to 237.5 mL sterile PBS

Cell count mixture

- 100 µL counting beads (concentration $3.0x10^5$/mL in 1 mL PBS/NCS/NaAcid)
- 40 µL PBS/FBS
- 10 µL Trypan blue
- 50 µL cells from pre-dilution

10x Phosphate Buffered Saline (PBS) 1L

- 85 g NaCl
- 2.50 g KH_2PO_4
- 6.85 g $NaHPO_4$

Add dH_2O to a total volume of 1000 mL

pH should be 7.4 ± 0.2

1x PBS/Tween 0.05% (5 L)

- Dilute 0.5 L sterile 10x PBS in 4.5 L dH_2O
- Add 2.5 mL Tween 20

PBS/BSA 0.14 %

- 50 mL sterile PBS
- 70 mg bovine serum albumin (BSA)

Filter through a 0.2 µg filter

1M H_2SO_4 (1L)

- 54.40 mL 18.4M sulphuric acid
- 949.50 mL ddH_2O

ELISA solutions	**ELISPOT solutions**
Di-sodium hydrogen phosphate 0.2 M (1L) • 28.30 g di-sodium hydrogen phosphate (Na_2HPO_2) 0.2 M Add ddH_2O to 1000 mL Citric acid 0.1 M (1L) • 21.01 g citric acid monohydrate ($C_6H_8O_7H_2O$) Add ddH_2O to 1000 mL Phosphate citrate buffer (pH 5.0 – 1L) • 257 mL 0.2 M Na_2HPO_2 • 243 mL 0.1 M $C_6H_8O_7 \cdot H_2O$ Add ddH_2O to 1000 mL OPD (Ortho-phenylediamine dihydrochloride) solution • Solve 10 mg OPD in 25 mL phosphate citrate buffer • Add 20 µL H2O2 immediately before adding to plate. Keep dark.	Sodium acetate 0.2 M (1L) • 27.2 g sodium acetate trihydrate (CH_3COONa) Add ddH_2O to 1000 mL, autoclave at 121°C for 15 minutes Acetic acid 0.2 M (1L) • 11.55 mL glacial acetic acid ($C_2H_4O_2$) Add ddH_2O to 1000 mL, autoclave at 121°C for 15 minutes Acetate buffer 50 mM (pH 5.0) 1L • 176 mL CH_3COONa • 74 mL $C_2H_4O_2$ • 750 mL ddH_2O Adjust pH to 5.0 ± 0.05 AEC (9-amino 3-ethyl carbazole) solution • Solve 20 mg AEC in 2.5 mL DMF • Add 47.5 mL acetate buffer • Filter through a 0.45 µm filter and add 25 µL 30 % H2O2 immediately before adding to plate. Keep dark.

2.11 Consumables

Needles, 23 G	BD Biosciences, USA
Syringe (vaccination) Micro-Fine ™ 0.3 mL (320830)	BD Biosciences, USA
Syringe 2 mL BD Plastipak 300186	Greiner Microlon, Germany
Filter 0.45 µm	Millipore, DK
Syringe Filter 0.20 µm	Whatman, UK
Microtubes 0.5 mL	Sarstedt, Germany
Microtubes 1.5 mL	Axygen Biosciences, USA
Elisa dilution tubes 1.3 mL (102270)	Greiner Bio-One, Germay

Paper towels for Elisa and Elispot	VWR International, Norway
Acetate foil for microtest well plates (82.1586)	Sarstedt, Germany
Pipettes	Thermo Labsystems
Thermo Labsystems Finnpipette Novus, multichannel	Termo Scientific, USA

3 Methods

3.1 Experimental protocol

3.1.1 Mice

Forty BALB/c mice (6-8 weeks old, female) were randomized and divided into two groups of 20 mice, named groups A and B. They were earmarked and randomly caged in groups of five animals. Both groups received one or two doses of an inactivated influenza H5N1 whole virus vaccine, group A intramuscularly and group B intranasally. Immunization, sampling and sacrifice were carried out as illustrated in figure 3.1.

All work with mice was carried out humanely according to The Norwegian Regulation on Animal Experimentation ("Forsøksdyrforskriften") and approved by the Animal Research Committee. They were acclimatized for one week and housed in pathogen-free surroundings, with 12 hour light/dark cycles in a temperature of 21°C. The daily care of the animals was performed by the personnel at the Vivarium, University of Bergen.

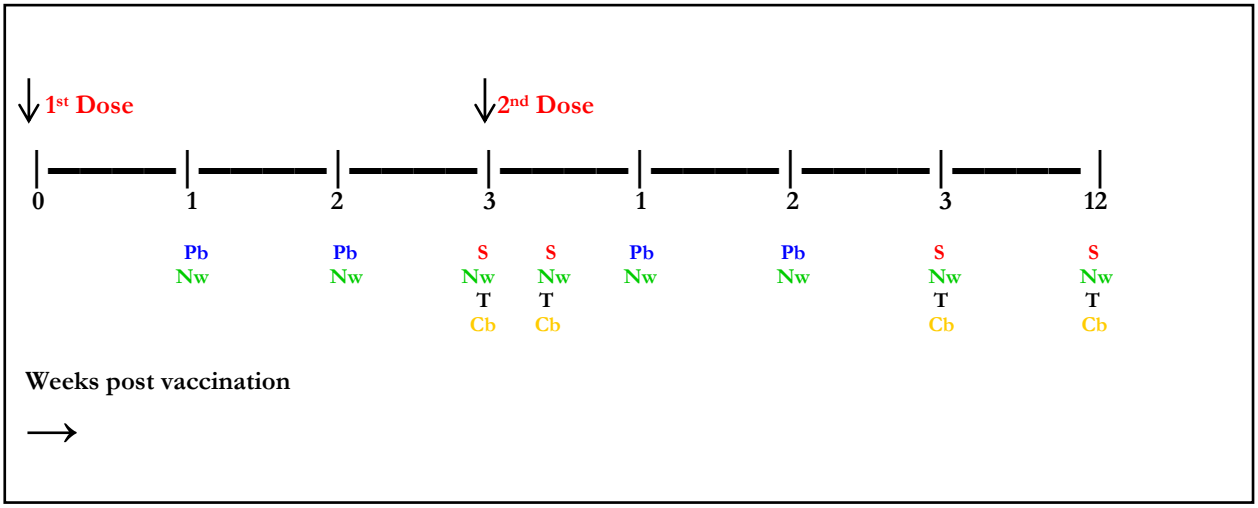


Figure 3.1. Immunization and sampling. Blood samples (Pb) and nasal wash samples (Nw) were collected once a week from groups of five mice. Three weeks after the first vaccination five mice from each group were euthanized, whereas the other mice got a second (booster) dose of vaccine. Mice were then sacrificed (s) four days and three weeks after the second immunization and cardiac blood (Cb) and tissue (T) (spleens and bone marrows) were collected. To see if the vaccine induced long lasting antibody responses, five mice from both groups were sacrificed twelve weeks after given the booster injection.

3.1.2 Vaccine

The virus vaccine strain (NIBRG-14) used in this study is produced by reverse genetics and is recommended by the WHO. It contains the HA and NA genes of the influenza A/Vietnam/1194/2004 (H5N1) virus and the internal genes of the vaccine donor strain PR8 (2:6 reassortant). The virus was propagated in embryonated hen's eggs and a whole virus vaccine was prepared and inactivated using β-propiolactone.

3.1.3 Intramuscular and intranasal immunization of mice

For intramuscular vaccination the mice were restrained in a plastic tube, the hind leg was held steadily and 50 µL of whole virus vaccine containing 7.5 µg HA was injected into the quadriceps muscles of the leg. In the other group, the animals were anesthetized to lie still. A 100-150 µL combination of Rompun Vet (1 mg/mL) and Ketalar (10 mg/mL) in sterile PBS was administered subcutaneously into the neck region. After approximately 15 minutes the mouse was checked for a suitable level of narcosis. The anaesthetized animals were laid on their back and the vaccine (40 µl, containing 7.5 µg HA) was administered by dripping 5 µl per nostril four times using a 20 µL pipette.

3.1.4 Collection of peripheral blood

Blood samples were obtained from the saphenous vein of the hind leg (Fig. 3.2). The animals were held steadily in a plastic tube with the hind leg strained. An area of fur was shaved with a scalpel and the vein was punctured using a 23 gauge needle. Blood (50-100 µL) was collected using a microvette capillary collection device. (The separation of sera from clotted blood is described below).

Figure 3.2. The vein used for sampling.

3.1.5 Collection of nasal wash samples

For nasal wash samples, the mice were held by the scruff of the neck upside-down over a petri dish (Fig. 3.3). The animals were rinsed from their mouth through the nostrils by drop wise administration in a steady stream using a 1 mL syringe with 350 µL PBS/BSA and a feeding tube. The exhaled nasal washings were collected from the petri dish and then pipetted into an eppendorf tube. Samples were kept on ice before freezing at -80°C.

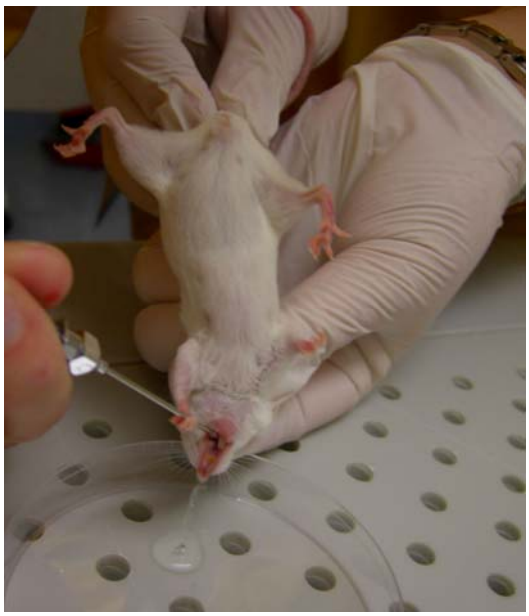


Figure 3.3. The nasal wash procedure.

3.1.6 Collection of cardiac blood and tissue

The mice were euthanized by CO_2-asphyxiation, and fastened with pins to a dissection plate. Immediately, the animals were exsanguinated by cardiac puncture and the blood was collected by using a 23 gauge needle and a 2 mL syringe. Next, animals were opened with sterile scissors from the abdomen to the throat and the skin were pulled aside and fixed. The spleen and one hind leg was aseptically removed and put into separate tubes containing sterile PBS.

3.1.7 Separation of sera

The blood samples were refrigerated at 4°C overnight, and serum separated from whole blood by centrifugation at 1000x g for 10 minutes. Cardiac blood samples were left at room temperature for 3-4 hours to clot before centrifugation for 10 minutes at 1000x g. Sera were transferred to microtubes and stored in the freezer at -80°C until testing.

3.2 Immunological assays

3.2.1 Isolation of lymphocytes

All work with lymphocytes was conducted aseptically in a laminar flow hood at room temperature.

Spleen: The spleen was placed in a petri dish and punctured several times at multiple sites using a 23 gauge needle and a 2 mL syringe with PBS/FBS gold (5 %) (Fig. 3.4). The procedure was repeated until the spleen turned greyish and was empty of cells. A total volume of 6 mL was transferred to a centrifugation tube.

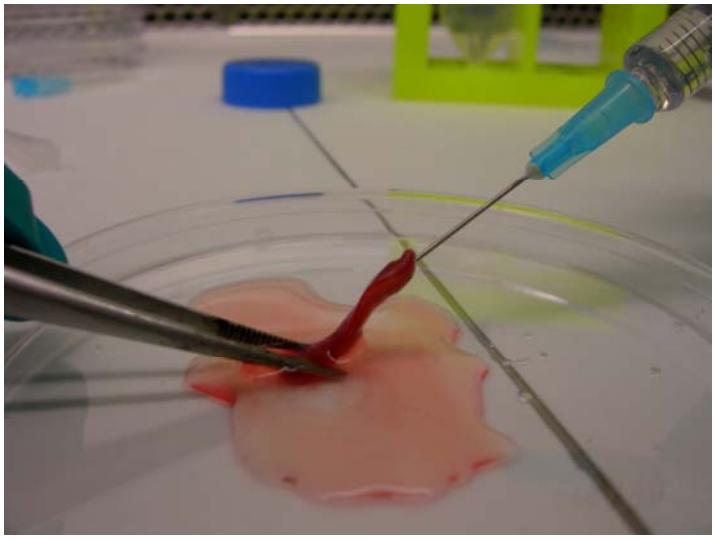


Figure 3.4. Isolation of splenocytes.

Bone marrow: Muscle tissue was removed from femur and tibia with sterile scissors and forceps. The two bones were separated in the knee joint and a small fraction from both ends of the bone was cut off. Each end of the bone was punctured and washed with 2 mL PBS/FBS, reversed and washed again three times. Lymphocytes (total volume of 6 mL) from both bones were transferred to a centrifugation tube.

Density gradient centrifugation with lymphoprep: The cell suspensions were carefully layered onto 3 mL of lymphoprep (in 15 mL tubes) using a Pasteur pipette. The tubes were centrifuged for 30 minutes at 800x g without brake at room temperature. Next, the white bands containing lymphocytes were removed and transferred into new tubes and cold PBS was added to a total volume of 8 mL. Tubes were centrifuged for 10 minutes at 250x g at 4°C and the supernatant was removed. Cells were re-suspended and in 8 mL of cold PBS and centrifuged again as described above. Finally, the lymphocytes were then re-suspended in 1 mL (bone marrow) or 2 mL (spleen) B-cell medium. The numbers of live lymphocytes were counted by flow cytometry. A 1:10 pre-dilution of cell suspension and PBS/FBS and a count mixture (description section 2.10) were made, and the number of live cells/mL was

determined by trypan blue exclusion. The concentration of lymphocytes was adjusted to $1.0x10^7$ cells/mL by adding B-cell medium.

3.2.2 Antibody ELISA

The enzyme-linked immunosorbent assay (ELISA) is an immunological technique which is used to detect antibodies specific for influenza in both murine sera and nasal wash samples. This method is based on the interaction between antigen and antibody, and by using secondary biotinylated antibodies, enzyme linked avidin with specificity for biotin, and a colorimetric substrate one can measure spectrophotometrically the concentration of antibodies present in a sample.

Elisa plates were coated with 100 µL/well of 2 µg/mL HA H5N1 whole virus influenza antigen or capture antibody (IgG, IgG1, IgG2a or IgA) diluted 1:1000 in PBS. After incubation at 4°C overnight, the plates were blocked with 200 µL PBS/NCS (20 %) for serum samples (150 µL for nasal wash samples) and incubated at room temperature for one hour. Next, 100 µL/well of each sample was added to the appropriate wells in 5-fold dilutions (serum, starting at 1:50, up to 1:781 250) or 2-fold dilutions (nasal wash, 1:5 up to 1:40) in duplicate. Furthermore, antibody standards from 50 to 0.781 ng/mL were diluted 2-fold and 100 µL added to each well in duplicate. Additional, two blanks containing 100 µL PBS/NCS were used to determine the background. The plates were incubated at room temperature for 1.5 hour for serum samples, and 2 hours for nasal wash samples. The plates were washed six times, three of them rapidly to avoid potential cross contamination and the last washes soaked the plates for a few minutes. Next, 100 µL/well of the appropriate goat-anti mouse specific-biotinylated antibodies (IgA, IgG and subclasses, 1:500) in PBS/NCS were added and incubated for one hour at room temperature. After six new washes, 100 µL/well of Extravidin Peroxidase in PBS/NCS (1:1000) were added and incubated at room temperature for one hour. Fresh OPD substrate (description section 2.10) was prepared and the plates were washed six times. H_2O_2 was added to the OPD-solution immediately before adding 100 µL/well to the plates. The reaction was stopped after 10 minutes incubation time with 100 µl/well of 1M H_2SO_4. The plates were read spectrophotometrically at 492 nm using the ELISA plate reader. Standard curves were obtained as log-log graphs in the Ascent program and the influenza specific antibody concentrations (ng/mL or µg/mL) after vaccination were calculated using standard curves and linear regression of the log transformed readings.

3.2.3 Antibody ELISPOT

The enzyme-linked immunospot assay is a method for detection of antibody secreting cells (ASC). Each ASC specific for influenza produces a spot and allows enumeration of the number of specific spots.

Ninety-six wells ELISPOT plates were coated aseptically with 100 µL/well of 2 µg/mL HA whole virus vaccine diluted in PBS, and incubated overnight at 4°C. The plates were emptied by flicking out the coating solution and blocked with 200 µL/well of PBS/FCS (5 %) and incubated in 5 % CO_2 at 37°C in a humidified incubator for one hour. The blocking buffer was removed and plates tapped carefully against some paper towels. One hundred µL of B-cell medium containing lymphocytes (100 000 to 500 000 cells/mL) from spleen or bone marrow was added to each well in duplicate and plates were placed in the CO_2 incubator at 37°C overnight. Plates were washed 6 times in PBS/Tween (0.05 %) with the Nunc Elisa washer, the first three washes were quick to avoid possible cross contamination, and the last three washes soaked the plates for a few minutes. To remove most of the washing solution the plates were tapped against paper towels between the washes. Next, 100 µL/well containing 2µg/mL goat anti-mouse biotinylated antibodies (IgA, IgM, IgG, or IgG subclasses), in PBS/T were added to the appropriate wells, and incubated two hours at room temperature. Following incubation, plates were washed six times as described above, before adding 100 µL to each well of Extravidin PO (1:1000) in PBS/T. After one hour incubation at room temperature the plates were washed six times before adding 100 µL/well of freshly made AEC substrate (description section 2.10) and the spots developed for 30 minutes at room temperature. The reaction was stopped by rinsing the plates under running tap water. Next, the plastic bottoms of the plates were removed and plates allowed to dry in the dark. The nitrocellulose membranes were removed and spots were counted using an Elispot reader and the Immunospot program.

3.2.4 Haemagglutination Inhibition Assay (HI-assay)

The influenza virus haemagglutinin agglutinates erythrocytes and by using a standardized amount of virus and red blood cells, the ability of serum antibodies in a sample to inhibit agglutination can be measured.

Sera were diluted 1:4 in receptor destroying enzyme (RDE), and incubated at 37°C overnight and further incubated for 30 minutes at 56°C to remove non-specific inhibitors and heat-inactivate the remaining RDE. Sera were then allowed to cool at room

temperature. Turkey red blood cells (RBC) were washed in cold PBS and centrifuged at 250x g for 10 minutes at 4°C. The procedure was repeated until the supernatant was clear (no haemolysed cells). Finally, a red blood cell suspension (0.7 %) was prepared by adding cold PBS.

3.2.4.1 Haemagglutination assay

In order to determine the virus titre, 50 µL PBS was added to each well of a V-shaped plate and 50 µL of virus suspension (whole virus) were added to the first row. The virus was double diluted by mixing and transferring 50 µL (to the next row). The final 50 µL was discarded. Red blood cells (0.7 %, 50 µL) was added to the well, mixed carefully, and incubated for 30 minutes at room temperature. The titre was read as the reciprocal of the virus dilution that gave 50 % agglutination, and adjusted to be 8 HA units (HAU)/50 µL.

3.2.4.2 Haemagglutination inhibition assay

V-shaped plates were added 50 µL PBS per well, in addition to 50 µL of the RDE treated sera in the first row. Starting with a 1:8 dilution duplicate, 2-fold dilutions were made and 50 µL of standardized virus solution (8 HAU) was added per well and incubated 1 hour at room temperature. Next, 50 µL turkey RBC was added and incubated at room temperature for 30 minutes. Finally, the plates were tilted and the haemagglutination inhibition titre was read as the reciprocal of the serum dilution that gave 50 % inhibition of haemagglutinin. For calculation purposes, negative values were given an arbitrary value of 4.

3.2.5 *In vitro* activation of lymphocytes

In order to determine the cytokine response from isolated splenic T-lymphocytes, the cells can be stimulated *in vitro* with the influenza antigen. This treatment activates the cells which instantly start to produce cytokines. After a longer period of incubation the supernatant can be analyzed and it is possible to distinguish between Th-1/Th-2 profiles.

Influenza activation medium were made by diluting influenza H5 virosomal vaccine in lymphocyte medium (final concentration; 10 µg HA/mL) and 100 µL/well was added to a 96-wells flat-bottom plate. Next, 100 µL/well of cell suspension containing $1.0x10^6$

lymphocytes were added to the plate. A mitogen medium consisting of phorbol myristate acetate (PMA), ionomycin and lymphocyte medium was used as positive controls, whilst negative controls consisted of medium alone (100 µL/well). Samples were incubated for 72 hours at 37°C in a humidified atmosphere of 5 % CO_2. The 200 µL from each well were then transferred to a V-shaped plate and centrifuged for 10 minutes at 300x g. The supernatants were removed, transferred to a new plate and frozen at -70°C until testing.

3.2.6 Multiplex bead assay

The multiplex bead assay can be used to measure a number of analytes in the same sample for instance cytokines, antibodies and RNA. By using polystyrene beads covered with antibodies and with differing colour intensity (red and infrared dyes), an instrument with a laser and a number of detectors is able to recognize multiple fluorescent signals.

The assay was used to determine the concentration of IL-2, IL-4, IL-5, IL-10, IL-17 and IFN-γ from the supernatants of *in vitro* activated splenic lymphocytes. The manufacturer's protocol was used. Standards were diluted 4-fold according to the protocol and placed on ice for 30 minutes. A multiscreen plate was pre-wet with 100 µL Assay Buffer A. Next, 120 µL stock beads were diluted in 2.88 mL Assay Buffer A, and 25 µL was added each well. The plate was washed with PBS/T (100 µL) twice by suction. Then, 50 µL from each standard, 25 µL of each sample diluted in 25 µL B-cell medium were added the appropriate wells and the plate was sealed and incubated in the dark at room temperature for 45 minutes on a platform shaker at 300 rpm. Detection Antibody (150 µL) was diluted in 2.85 mL Detection Antibody Dilutent, the plate was washed three times by suction and 25 µL was added each well. The plate was incubation as described above for 30 minutes. After three washes, Streptavidin-PE (30 µL) diluted in 2.97 mL Assay Buffer A was added to the plate (25 µL/well) and incubated for 10 minutes. Before reading by the Luminex instrument, the plate was washed and Assay Buffer A added (100 µL/well). A standard curve (Bioplex manager 5) was obtained and cytokine concentration determined.

3.3 Statistical analysis

To compare the two groups of mice, statistical analyses were performed using SPSS 15.0 for Windows and the results (ELISA, ELISPOT and multiplex assay) were analyzed by

using a two-sided Student's *t*-test assuming equal variances. *P*-values ≤ 0.05 were considered significant. The HI results were analyzed using Prism 5.0 for Windows (GraphPad software) with a ± 95% confidence interval.

4 Results

In this study we examined the local and systemic humoral immune response in BALB/c mice vaccinated with whole inactivated influenza A/Vietnam/1194/2004 (H5N1) virus vaccine. We also examined the T cellular responses by studying the cytokine profiles after the second vaccination. Two groups of 20 mice were intramuscularly or intranasally vaccinated with two doses of 7.5 µg HA (half of the normal human dose). Thus in this study we directly compared the immunological profiles induced by the two administration routes. The two groups will be referred to as the intramuscular (IM) group and intranasal (IN) group according to the route of vaccine administration. The study also included a control group of 10 animals, which only received PBS.

Different immunological assays were used to assess the immune response in the serum, the nasal wash, spleen and bone marrow. The results presented in this section are from eight different sampling days of five mice in each group. The two vaccine doses were given 21 days apart and serum samples and nasal washes were collected each week, including 4 days post second vaccination. Spleens and bone marrow were removed after euthanasia. (An overview of the time points of vaccination and sampling can be found in the method section 3.1.1)

4.1 The humoral immune response induced after vaccination

The humoral immune response induced after intramuscular or intranasal vaccination was investigated using a range of immunological assays. The concentration of serum antibodies (IgG, IgG1, IgG2a and IgA) and nasal wash (IgA) was quantified using ELISA. Moreover, ELISPOT was used to detect the influenza specific (IS) antibody secreting cells (ASC) for the classes IgM, IgA and IgG, including the IgG1 and IgG2a subclasses. The Haemagglutination Inhibition (HI) assay was used to analyze the serum anti-HA response.

4.1.1 Two doses of whole inactivated virus vaccine induced high antibody titres

The HI assay was conducted using turkey erythrocytes for measuring anti-HA antibodies. Serum samples from euthanized mice collected after the first dose (3 weeks) and after the second dose (four days, three weeks, and twelve weeks) were investigated. No HI antibodies (titres <8) were detected in the serum from the control mice (results not shown). For all other samples, an HI titre <8 was assigned an arbitrary value of 4 for calculation of geometric mean titres (GMT).

Only low HI-titres were found in the IM group after one dose of vaccine, with only two of the five mice having detectable HI antibody in this group (titres of 32 and 48, respectively) (Fig. 4.1). No HI antibody was detected in the IN group, all mice had HI titres of <8. The second vaccination significantly boosted the response, and by day four all mice had detectable antibodies, ranging from a titre of 64 to 384 (IM group) and 48 to 256 (IN group). The highest titres were observed three weeks after the second dose with geometric mean HI titres of approximately 370 for the IM group, and 230 for the IN group, respectively. There were no significant differences in HI antibody response between the two administration routes. HI antibodies were still detectable in all mice in both groups by twelve weeks after the second dose, although the geometric mean titres for the groups had declined to mean titre 148 (IM) and 115 (IN).

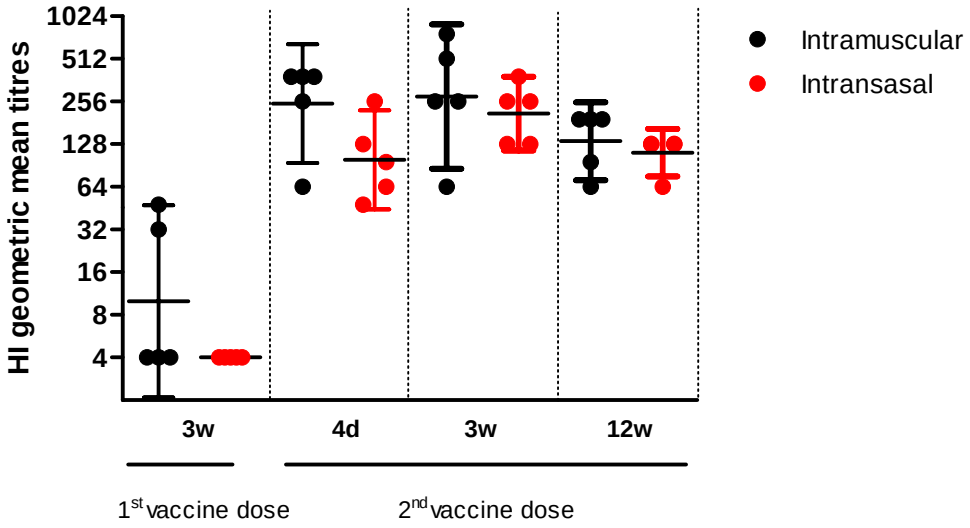


Figure 4.1. The haemagglutination inhibition (HI) antibody response induced after intramuscular (IM) and intranasal immunization (IN) with influenza H5N1 virus. BALB/c mice were IM (black circles) or IN (red circles) vaccinated with one or two doses (3 weeks apart) of 7.5 µg HA influenza A/Vietnam/1194/2004 (H5N1) whole virus vaccine**.** The data are presented as the geometric mean titre (GMT) from five mice in each group on four different sampling days. Each circle represents titres from one mouse. The data are presented as the geometric mean titre ± 95% confidence interval. The point 4d on the x-axis indicates four days after 2nd dose, W = weeks.

4.1.2 Two doses of intramuscular vaccine induced high serum IgG levels

No influenza specific antibodies were detected in the control mice at any time point (results not shown). Vaccination induced only very low concentrations of influenza IgG specific antibodies in the IN group after one week (0.3 µg/mL); whereas a significantly higher ($p<0.05$) response was observed in the IM group (6.2 µg/mL). The antibody concentration increased in both vaccine groups over the next two weeks with the IM group showing a 10-fold higher level than the IN group. After the second dose, the concentrations of IgG significantly increased in the IM group reaching a peak level of 2000 µg per mL three weeks after the second vaccine dose (Figure 4.2).

Although the antibody concentration of the IN group also increased after the second dose, it was significantly lower ($p<0.05$) than the concentration in the IM group. Similarly to the IM group, the IgG response in the IN group also reached a peak three weeks after the second vaccination with a mean antibody concentration of 770 µg per mL. Twelve weeks after the second injection, the vaccine still elicited a high antibody response in the two

groups. The IgG concentration in the group vaccinated intramuscularly was approximately 1100 µg per mL, thus a 50 % decline from the peak response. In contrast, in the mice receiving the intranasal vaccine the concentration had declined to 400 µg per mL, a 57 % decline from the peak response (Fig. 4.2).

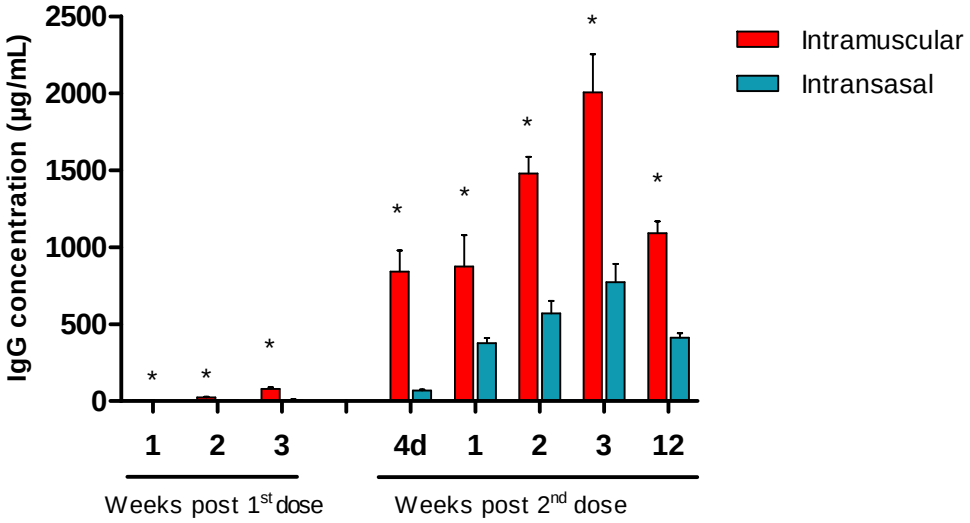


Figure 4.2. The serum IgG response induced after intramuscular (IM) and intranasal (IN) immunization with H5N1 virus. BALB/c mice were IM or IN vaccinated with one or two doses of 7.5 µg HA influenza A/Vietnam/1194/2004 (H5N1) whole virus vaccine. The IgG serum concentrations are shown in the group of mice vaccinated intramuscularly (red bars) and the group vaccinated intranasally (blue bars). The point 4d on the x-axis indicates four days after 2nd dose. The columns represent the mean antibody concentration in microgram per millilitre (µg/mL) calculated from groups of five mice. The error bars represents the standard error of the mean (SEM). The IM group had statistically significant higher ($p<0.05$) concentrations of IgG than the IN group and are indicated by an asterisk (*).

Two doses of whole inactivated virus vaccine induced high serum IgA levels in intranasally vaccinated mice

No IgA antibody was detected in the control group (results not shown), or after the first intramuscular injection. However, by four days post second IM dose the antibody concentration increased to 330 ng/mL, and this concentration remained essentially stable until the end of the experiment (Fig. 4.3). Twelve weeks after the second dose, the highest concentration of IgA was found with 374 ng IgA per mL, thus a 12 % increase from day four.

Although no detectable antibodies were found one week post vaccination, the highest concentrations of IgA were observed in the group that received two doses of the intranasal vaccine. Over the next two weeks the concentration started to increase, and by three weeks after the second immunization there was a peak response of approximately 5400 ng per mL. This mirrors the serum IgG response described above where the peak response was also found three weeks post the second dose. Twelve weeks after the second immunization, serum IgA was still detected although concentration had fallen to nearly 3000 ng/mL (Fig. 4.3), almost half of the concentration calculated at the peak response.

A minor decrease in serum IgA concentrations was observed in both experimental groups two weeks after the second dose, which may have been due to differences in the groups of mice sampled.

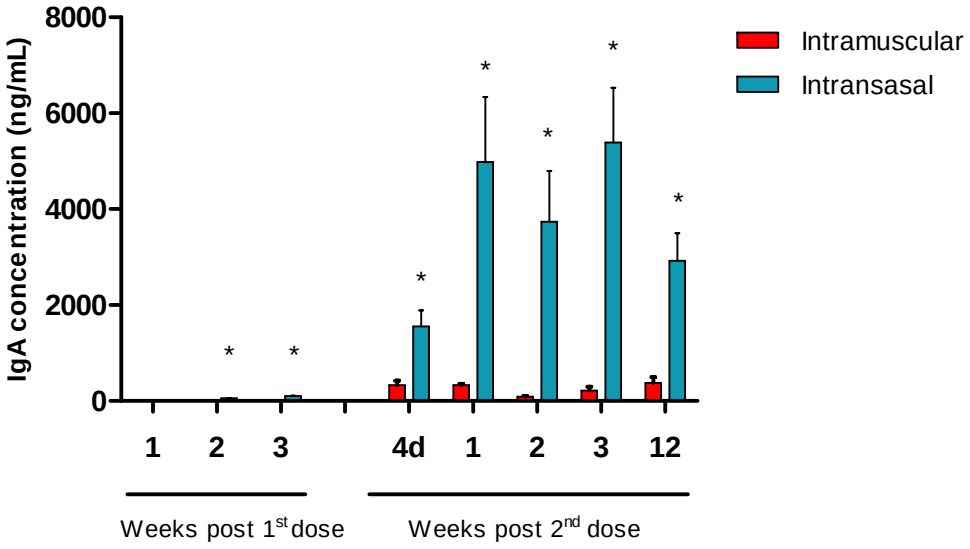
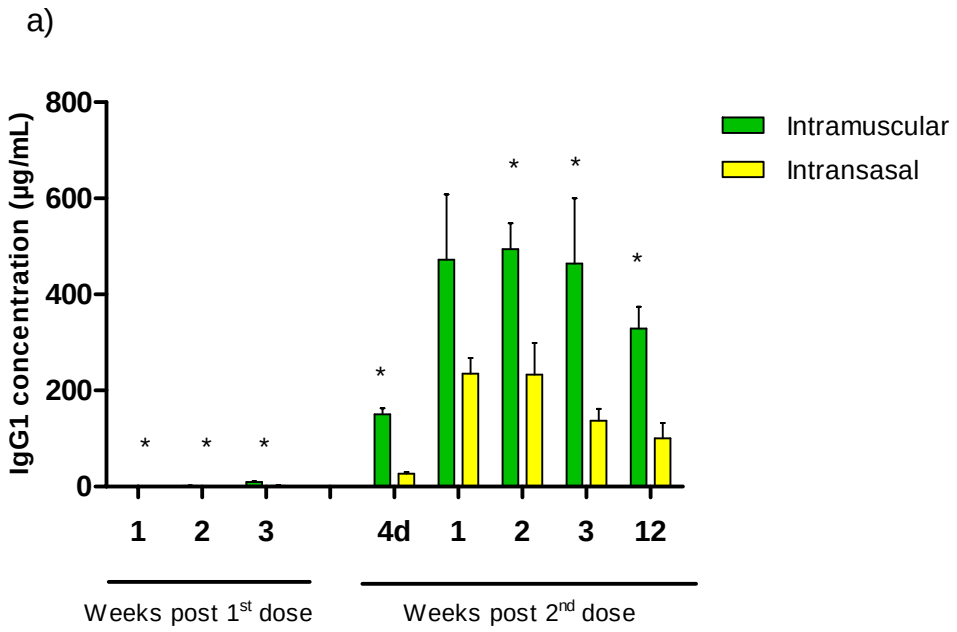


Figure 4.3. The kinetics of the serum IgA response induced after intramuscular (IM) and intranasal (IN) immunization with influenza H5N1 virus. BALB/c mice were IM or IN vaccinated with one or two doses (3 weeks apart) of 7.5 μg HA influenza A/Vietnam/1194/2004 (H5N1) whole virus vaccine**.** The bar graph illustrates the serum IgA antibody response of the IM group (red bars) and the IN group (blue bars) at various time points after one or two doses of influenza vaccine. The mean antibody concentrations from groups of five mice are presented in nanogram per millilitre (ng/mL). Error bars are the standard error of the mean (SEM). The point 4d on the x-axis indicates four days after 2nd dose. The IN group had statistically significant higher concentrations of IgG than the IM throughout the experiment ($p < 0.05$) indicated by an asterisk (*).

To sum up, the two groups both elicited high HI titres after two vaccine doses and there were no significant differences between the vaccine routes. Moreover, after analyzing the serum antibody classes, we found that the IM group had significantly higher concentrations of IgG, whereas the IN group had significantly higher concentrations of IgA.

4.1.3 Analysis of the serum IgG subclass response after vaccination

The two IgG antibody subclasses IgG1 and IgG2a which can be used as markers for a Th-1 (IgG2a) or a Th-2 (IgG1) response were examined. Very low concentrations (0.6 µg/mL) of serum IgG1 were detected the first week after immunization in the IM group, whereas no IgG1 was detected in the IN group (Fig. 4.4a). Over the next two weeks the concentrations remained low, however it was significantly higher ($p<0.05$) in the IM group (9.4 µg/mL) than in the IN group (1.8 µg/mL). Four days after the second dose of vaccine a significant increase ($p<0.05$) in IgG1 concentrations were observed for both vaccine groups. The IN group had a peak response with a concentration of 235 µg/mL one week after the second dose, whilst the response in the IM group peaked a week later with approximately 495 µg/mL.



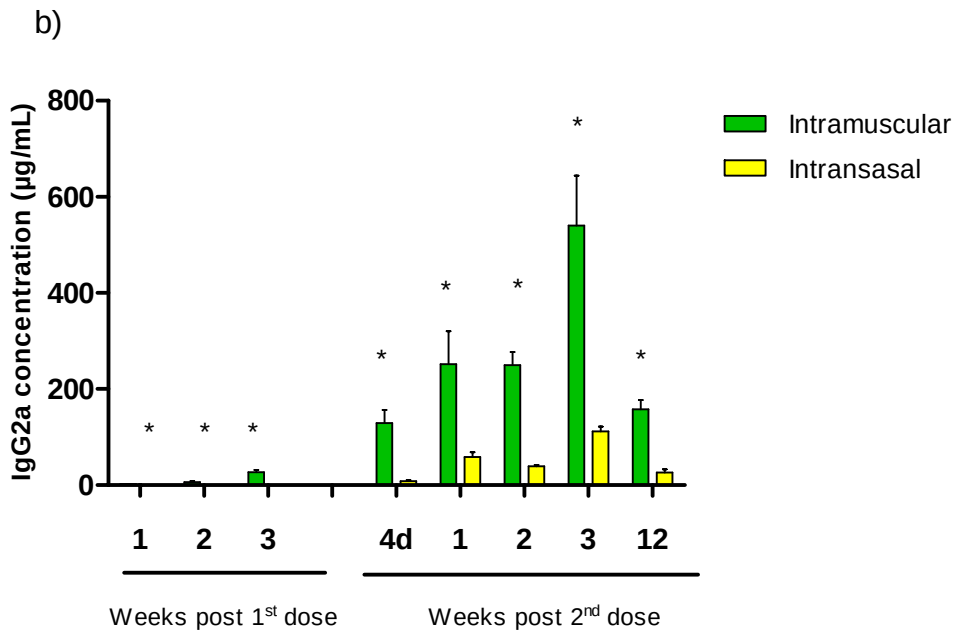


Figure 4.4. The influenza-specific serum IgG1 and IgG2a subclasses after intramuscular (IM) and intranasal immunization (IN) with influenza H5N1 virus. BALB/c mice were IM or IN vaccinated with one or two doses of 7.5 µg HA influenza A/Vietnam/1194/2004 (H5N1) whole virus vaccine**.** The figures depict serum antibody concentrations of a) IgG1 and b) IgG2a. Green bars are the IM group, and yellow bars are the IN group. The columns represent the mean concentration from five mice measured in microgram per millilitre ±standard error of the mean (SEM) at different sampling points. The point 4d on the x-axis indicates four days after 2nd dose. The concentrations of IgG1 and IgG2a were significantly higher in the IM group than in the IN group throughout the study, except for IgG1 one week after second vaccine dose ($p < 0.05$), indicated by an asterisk (*).

Twelve weeks post the second vaccine dose the IM group still had significantly higher ($p<0.05$) antibody concentrations of IgG1 (330 µg/mL) than the IN group (100 µg/mL) (Fig. 4.4a).

We also investigated the induction of IgG2a after vaccination (Fig. 4.4b). IN immunization elicited a somewhat lower IgG2a antibody response than IgG1. No antibodies were detected during the first week after vaccination and the concentration of IgG2a remained low (≤0.5 µg/mL) over the next two weeks. Four days after the second immunization the concentration of IgG2a increased to 8.0 µg/mL. Subsequently the concentration increased considerably, and peaked three weeks after the second dose (approximately 112 µg/mL).

In the group vaccinated intramuscularly, IgG2a was detected one week after the first dose (2.0 µg/mL) and the concentration of IgG2a continued to increase up to three weeks (27 µg/mL) (Fig. 4.4b). Here, the IM group showed significantly higher concentrations than the IN group. The second vaccination boosted the response by day four (approximately 130 µg/mL) with a peak response observed three weeks after immunization (540 µg/mL). Twelve weeks after the second dose the concentrations had declined to 158 µg/mL in the IM group which was significantly higher than the IN group (27 µg/mL) (Fig. 4.4b).

4.1.4 The subclass distribution after vaccination

The distribution of IgG1 and IgG2a subclasses elicited after the two routes of vaccination was also compared. The IgG2a/IgG1 ratio is an indicator of the T-helper response, with a ratio over 1.0 indicating a T-helper 1 profile, and below 1.0, a T-helper 2 profile.

After one dose of vaccine there was a dominant IgG2a response (Th-1 like) in the IM group (IgG2a/IgG1 ratio 3.7 – 2.9) (Fig. 4.5). After the second vaccination the profile shifted to a Th-2 response with a ratio of 0.8 four days after the second dose, and declined further to 0.5 during the next week. However, by three weeks post the second dose we found a balanced Th1/Th2 distribution (IgG2a/IgG1 ratio approximately 1.1), which at the end of the study and had fallen to 0.5 (Th-2-like).

In contrast, the IN group had an overall dominant IgG1 subclass. The IgG subclasses were not detected until two weeks after immunization (IgG2a/IgG1 ratio 0.6). The concentration of IgG1 antibodies increased and throughout the remainder of the time period the ratio was stabile around 0.2-0.3, except for a slight increase three weeks after the second immunization (0.8).

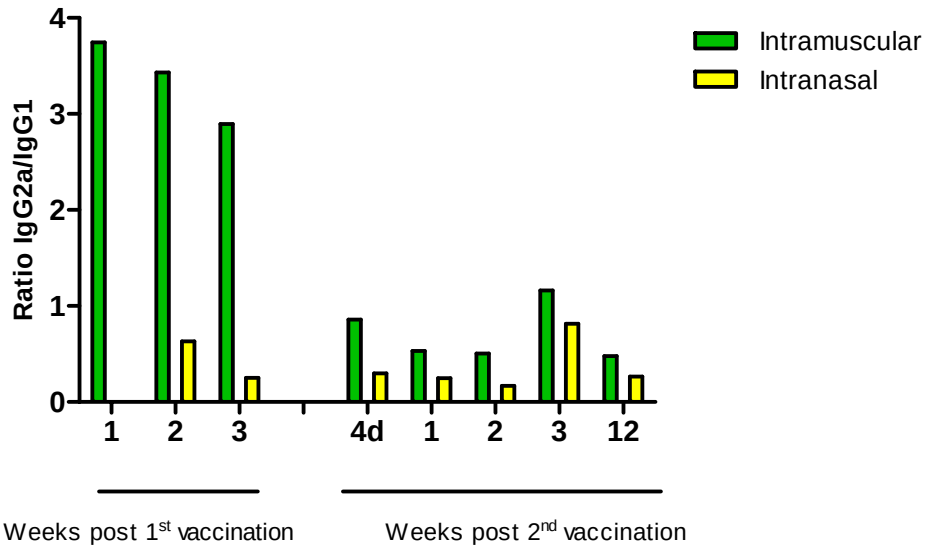


Figure 4.5. Distribution of the IgG subclasses after intramuscular (IM) and intranasal (IN) immunization with influenza H5N1 whole virus. BALB/c mice were IM or IN vaccinated with one or two doses of 7.5 μg HA influenza A/Vietnam/1194/2004 (H5N1) whole virus vaccine. The values on the y-axis show the ratio between the two subclasses (serum IgG2a concentration divided by serum IgG1 concentration). Green bars are the intramuscularly vaccinated mice (IM), whereas yellow bars represent the intranasally vaccinated mice (IN). The point 4d on the x-axis indicates four days after 2nd dose.

The results generally show a dominant IgG2a serum antibody in the IM group, which is characterized by a Th-1 subclass distribution after one dose and a more mixed profile after the second vaccine dose. In contrast, the IN group was dominated by IgG1 serum antibody and a Th-2 distribution after both one and two doses.

4.1.5 Two intranasal vaccinations induce high mucosal IgA levels in the nasal cavity

We investigated the local immune response in the nasal cavity by collecting nasal washes at various time points after vaccination (Fig. 4.6). Only IgA antibody concentrations at or just above the detection limit (4 to12 ng/mL) were found in the nasal washes after one dose of vaccine in both groups. After the second dose, the concentration rose significantly in the IN group from 192 ng/mL after one week to a peak response of 554 ng/mL at two weeks post vaccination. The concentration rapidly declined by three weeks to 232 ng/mL, and after twelve weeks 120 ng/mL was measured. In contrast, the concentration of IgA in the

IM group remained just above the detection limit throughout the entire experiment (ranging from 6 to 12 ng/mL).

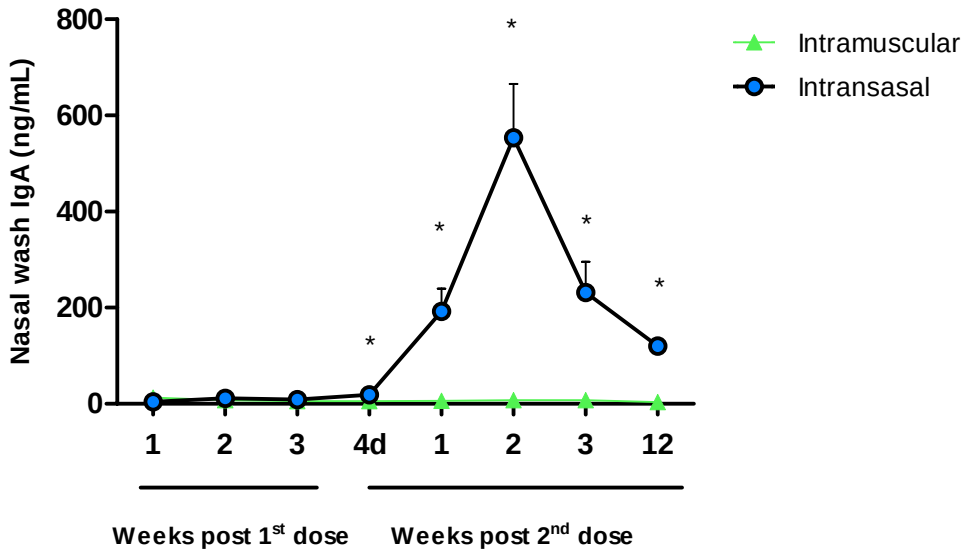
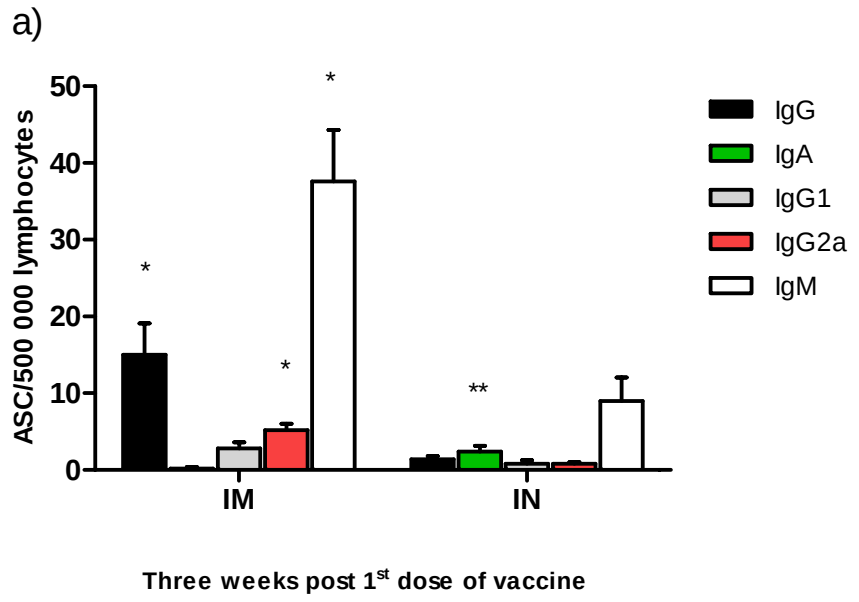


Figure 4.6. The mucosal IgA immune response after intramuscular (IM) and intranasal (IN) immunization with influenza H5N1 virus. BALB/c mice were vaccinated with one or two doses of 7.5 µg HA influenza A/Vietnam/1194/2004 (H5N1) whole virus vaccine IM or IN**.** The graphs show the mean concentration of IgA in nasal wash collected at eight time points. The green triangles are the intramuscularly vaccinated mice (IM), the blue circles represents the intranasally vaccinated mice (IN). The concentrations are presented as nanogram per millilitre (ng/mL) ±SEM. The last point in the IN group (12 weeks) is calculated from four mice. The concentrations of IgA in the IN group were significantly higher ($p<0.05$) than group IM after the second vaccination, indicated by an asterisk *.

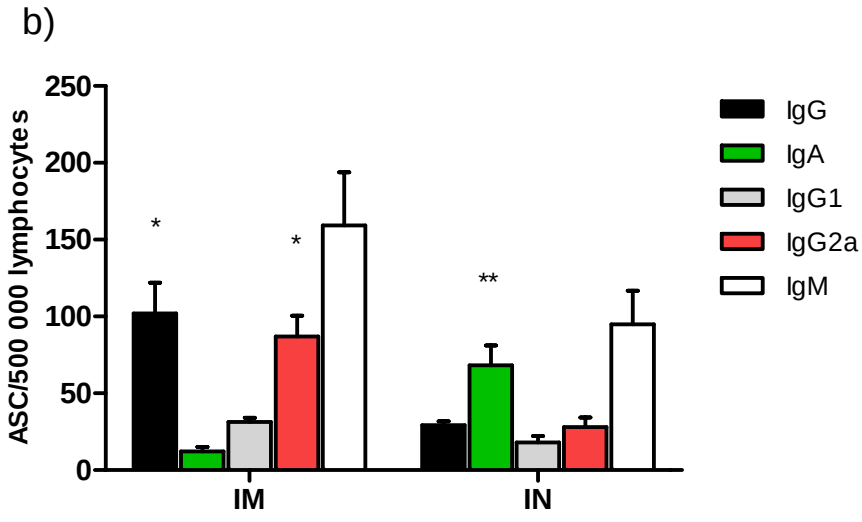
4.1.6 The influenza specific antibody secreting cell (ASC) response elicited in the spleen

The ELISPOT assay was conducted to enumerate the number of influenza specific ASC three weeks after the first vaccination, four days, and three weeks after the second vaccination. In the control mice a number of IgM ASC was detected (mean 24 (range 19-30) ASC per 500 000 lymphocytes) and was subtracted from the results of the two experimental groups. The results of IgM ASC from the control mice are not included in the graphs. No ASC from the other classes or IgG subclasses were found in the control mice.

After the first dose of vaccine, influenza specific IgM ASC dominated in the IM group (mean number 38), followed by IgG ASC (15) (Fig. 4.7). Only low numbers of IgG1 and IgG2a were detected, the latter subclass in somewhat higher numbers. In the IN group, there were low numbers of all antibody classes with the following distribution; IgM > IgA > IgG > IgG1 = IgG2a (Fig. 4.7a). In the IM group the numbers of IgM, IgG and IgG2a were significantly higher group than the IN group, whereas IgA were significantly higher in the IN group.



Three weeks post 1st dose of vaccine



Four days post 2nd dose of vaccine

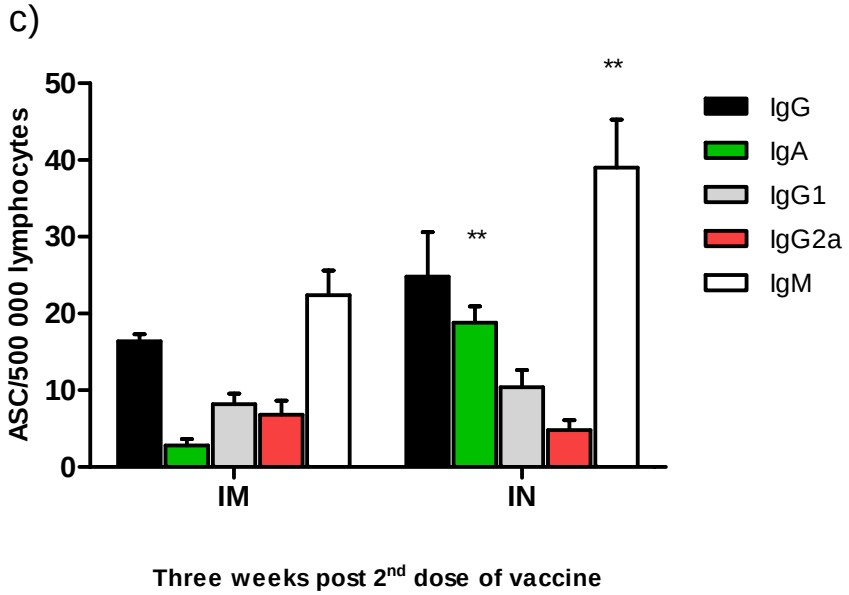


Figure 4.7. Influenza specific antibody secreting cells (ASC) in the spleen at various time points after immunization. BALB/c mice were intramuscularly (IM) or intranasally (IN) vaccinated with one or two doses of 7.5 µg HA influenza A/Vietnam/1194/2004 (H5N1) whole virus vaccine. The data are presented as the mean number of ASC per 500 000 lymphocytes ±SEM. IgG (black bars), IgA (green bars), IgG1 (grey bars), IgG2a (red bars) and IgM (open bars). a) ASC response three weeks after one dose of vaccine, b) ASC response four days after two doses of vaccine. IgG1 ASC in the IN group were counted from only one mouse due to difficulties in the spot counting program. In the IM group the IgG results were counted from four mice, and the IgG1 results from three mice. c) ASC response three weeks after two doses of vaccine. Significantly higher numbers ($p<0.05$) are indicated by asterisks: * = significantly higher than IN, ** = significantly higher than IM.

After the booster dose the ASC numbers were significantly higher ($p<0.05$) (Fig. 4.7b). Four days after the second dose the highest numbers of influenza specific ASC were found in the IM group although the number of IgA ASC in the IN group was significantly higher than the IM group (IM; mean number 12 and IN 68). The numbers of IgG and IgG2a were significantly higher in the IM group, which had had the following distribution; IgM > IgG > IgG2a > IgG1 > IgA. The IN group in contrast showed; IgM > IgA > IgG > IgG2a > IgG1.

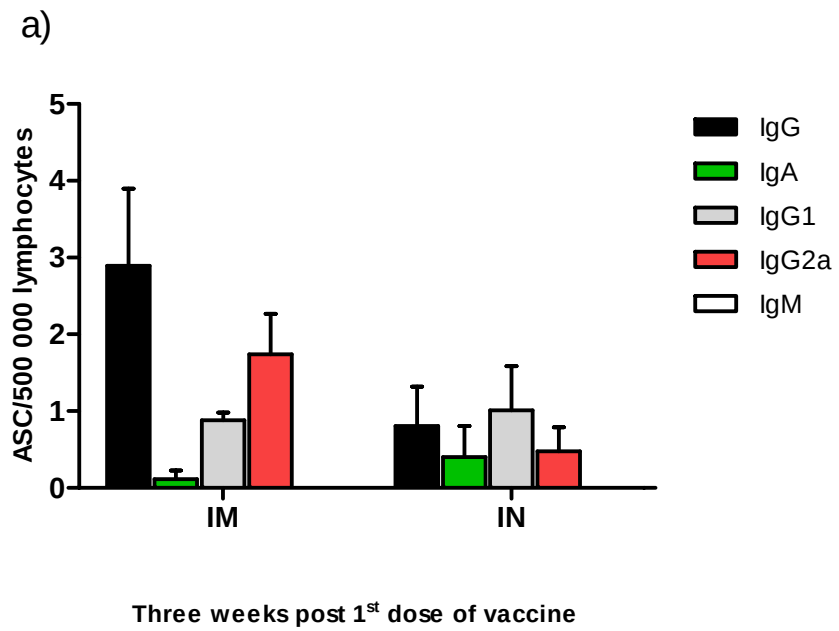
Three weeks after the booster dose the numbers of influenza specific ASC had significantly decreased ($p<0.05$) (Fig. 4.7c). At this time point, the numbers were now

highest in the IN group, except for IgG2a which was slightly higher in the IM group. The IgA ASC and IgM were significantly higher in the IN group than in the IM group. The distribution of the classes and subclasses in the IM group was the same as observed at day four, however the IgG1 numbers were slightly higher than IgG2a numbers. The IN group now had the following distribution; IgM > IgG > IgA > IgG1 > IgG2a.

4.1.7 The influenza specific antibody secreting cell (ASC) response elicited in the bone marrow

The ASC in the bone marrow was analyzed at the same time points as in the spleen. The control mice had low numbers of IgM ASC, (mean 8 ASC per 500 000 lymphocytes) but not other classes or subclasses and this background was subtracted the specific response in the two groups.

Three weeks after the first immunization only very low numbers of influenza specific ASC were detected in both groups (for all antibody classes; range 0-6 ASC per 500 000 lymphocytes) (Fig. 4.8a).



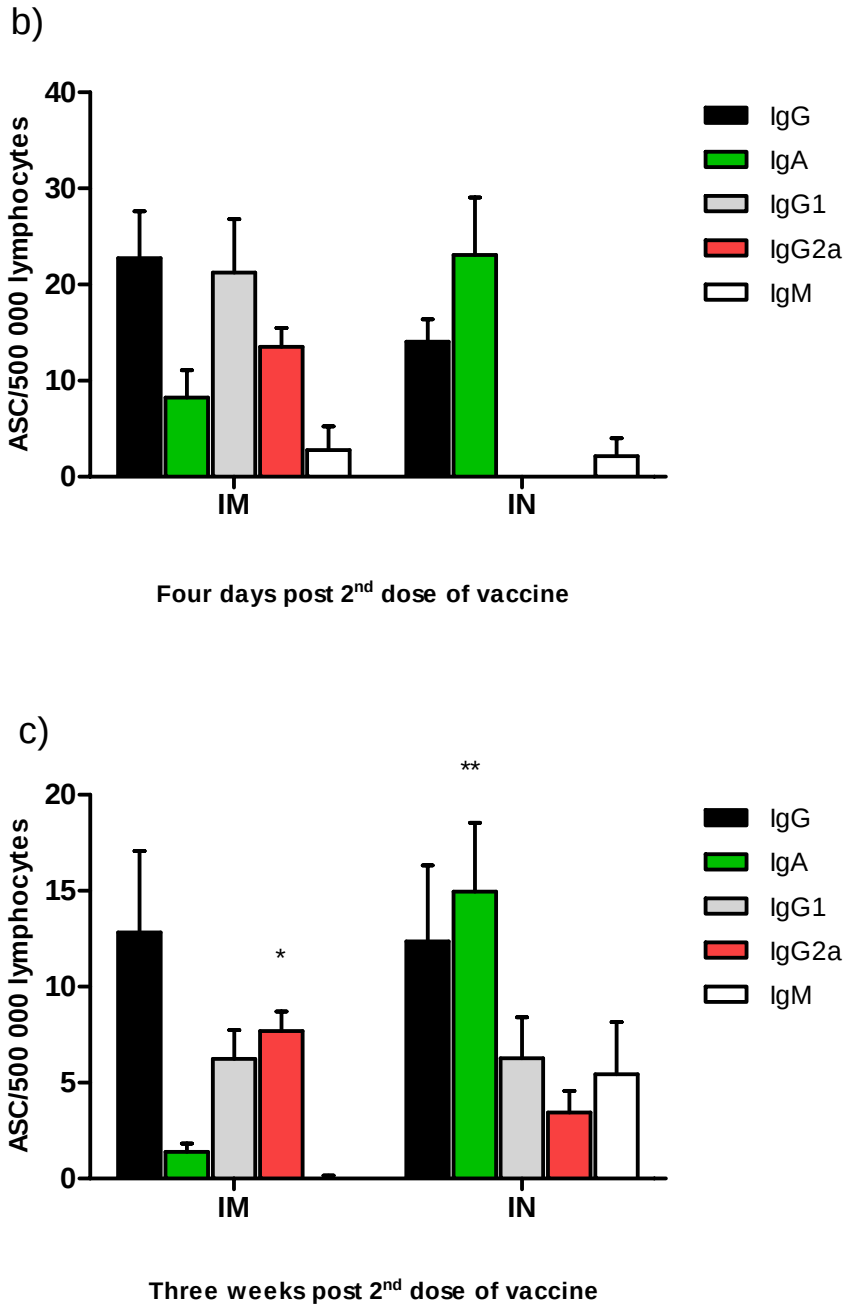


Figure 4.8. The influenza specific antibody secreting cells (ASC) in the bone marrow at various time points after immunization. BALB/c mice were intramuscularly (IM) or intranasally (IN) vaccinated with one or two doses of 7.5 μg HA influenza A/Vietnam/1194/2004 (H5N1) whole virus vaccine. The data are presented as the mean number of ASC per 500 000 lymphocytes ± standard error of the mean (SEM) from five mice in each group at each time point. IgG (black bars), IgA (green bars), IgG1 (grey bars), IgG2a (red bars) and IgM (open bars). a) ASC response three weeks after one dose of vaccine. The IgA and IgM results are only calculated from four mice b) ASC response four days after two doses of vaccine. The ASC response in the IN group was only analyzed for IgG, IgM and IgA, and the IgG results are from four mice due to difficulties in the spot counting program. c) ASC response three weeks after two doses of vaccine.

Significantly higher numbers ($p<0.05$) are indicated by asterisks: * = significantly higher than IN, ** = significantly higher than IM.

The second vaccination significantly boosted the response, by four days post second vaccination. The group vaccinated intramuscularly had higher numbers of IgG ASC than the group vaccinated intranasally, which had a higher number of IgA ASC (Fig. 4.8b). This mirrors the results of ASC detected in the spleen at the same time. The distribution of ASC in the IM group was as follows; IgG > IgG1 > IgG2a > IgA > IgM, and in the IN group; IgA > IgG > IgM. The IgG1 and IgG2a response was not analyzed at this point (Fig. 4.8b).

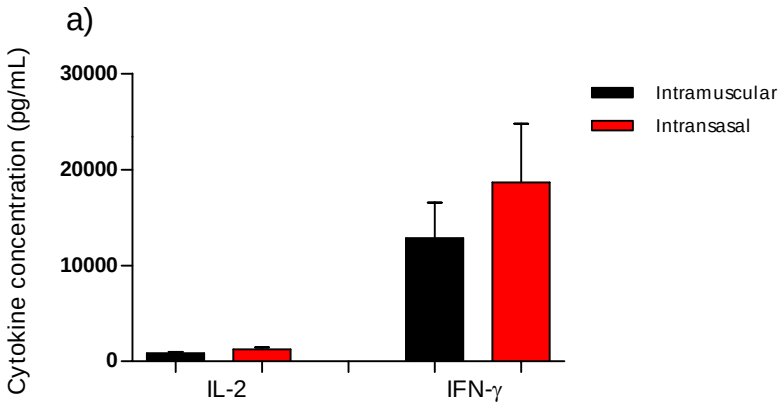
Three weeks after the second vaccination, the numbers of influenza specific ASC decreased (Fig. 4.8c). All Ig classes were still detected in both experimental groups, except for IgM ASC in the IM group. The mean IgG ASC numbers in the two groups were almost the same (IM 12.8 and IN 12.4 ASC per 500 000 cells), and were somewhat lower than seen in the spleen. The intranasally vaccinated mice had significantly higher numbers of IgA ASC, similarly to the results from the spleen. The distribution of the antibody classes were; IgG > IgG2a >IgG1 > IgA in the IM group, and IgA > IgG > IgG1 > IgG2a > IgM in the IN group (Fig. 4.8c). This contrasts with the splenic response which was dominated by the IgM ASC in both groups.

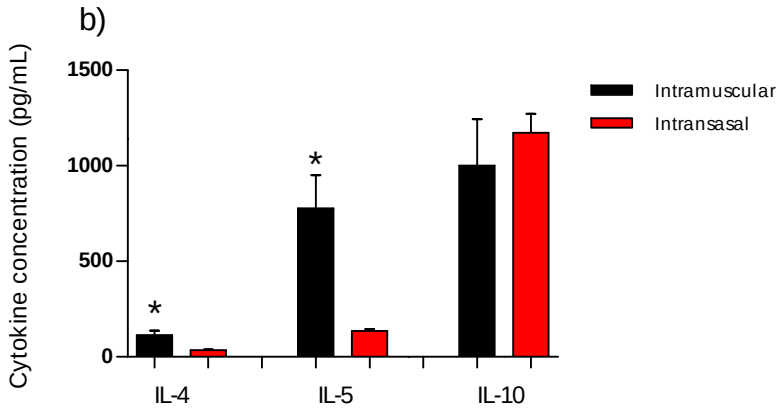
To summarize the ELISPOT results, the intranasally vaccinated mice in general displayed higher numbers of IgA ASC than the intramuscularly vaccinated mice in both the spleen and the bone marrow at all time points. The IM group, on the other hand, had higher numbers of IgG ASC, with the exception of the splenic response four days post second vaccination.

4.2 The cytokine response from *in vitro* activated splenocytes

The multiplex bead assay was used to investigate the splenic cytokine responses after vaccination. The test was conducted three weeks after the second vaccine dose to investigate the T helper profiles of the two groups. We measured the concentrations of IL-2 and IFN-γ (Th-1 markers), IL-4, IL-5 and IL-10 (Th-2 markers) and IL-17 (Th-17 marker) in the supernatants of *in vitro* stimulated splenocytes.

The cells were incubated for 72 hours with H5 vaccine diluted in medium and negative controls were incubated with medium alone. The concentration of cytokines in the supernatant of the negative controls was subtracted from the cytokine concentration of the stimulated cells.





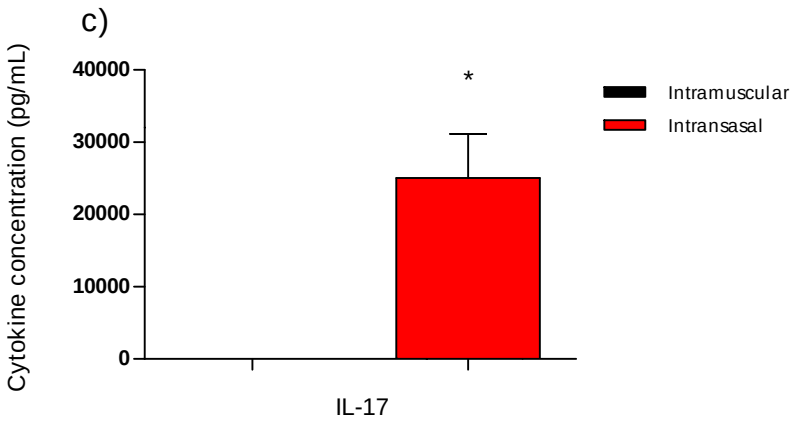


Figure 4.9. The cytokine response from *in vitro* activated splenic lymphocytes three weeks after second vaccination. BALB/c mice were intramuscularly (IM) or intranasally (IN) vaccinated with one or two doses of 7.5 µg HA influenza A/Vietnam/1194/2004 (H5N1) whole virus vaccine. The bar graphs depict the mean cytokine concentration in picogram per millilitre (pg/mL) from five mice. Black bars represent intramuscular vaccinated mice (IM) and red bars intranasally vaccinated mice (IN). Error bars are the ±standard error of the mean (SEM). Isolated splenic lymphocytes were *in vitro* activated with Influenza H5N1 virosomal vaccine and the supernatants from one million cells/well were analyzed for secreted cytokines. a) IL-2 and IFN-γ, b) Il-4, IL-5 and IL-10, c) IL-17. Significantly higher concentrations ($p>0.05$) are indicated by an asterisk (*).

Both vaccine groups secreted the Th-1 cytokines IFN-γ and IL-2, where IFN-γ was secreted in a much higher concentration than IL-2. There were no significant differences between the two groups (Fig. 4.9a). In the IM group, the concentrations of the Th-2 cytokines IL-4 and IL-5 were significantly higher than in the IN group which only secreted low concentrations. The concentration of IL-10 was almost similar (IM; 1002 pg/mL and IN; 1173 pg/mL) in the two groups (Fig. 4.9b). We also measured the secretion of IL-17 and found that the IN group secreted high concentrations this cytokine, but it was not secreted by the IM group (Fig. 4.9c).

To sum up all results, the HI titres of both groups were high after two vaccine doses. The intramuscularly administered vaccine had high concentrations of serum IgG, whereas the intranasally administered vaccine had high concentrations of serum IgA and also local IgA after two vaccinations. The ASC numbers were higher in the spleen than in the bone marrow and generally, the IM group had higher numbers of IgG ASC and the IN group had higher numbers of IgA ASC. Furthermore, the IM group was dominated by IgG2a after one vaccine dose and more mixed IgG2a/IgG1 subclass distribution after two doses. This

group secreted significantly higher concentrations of IL-4 and IL-5 than the IN group and both groups secreted high concentrations of IL-10. The IN group secreted high concentrations of IL-17, but also IFN-γ.

5 Discussion

Within the last decades a number of influenza A viruses of avian and swine origin (H5N1, H7N7, H9N2 and H1N1) have crossed the species barrier and caused infection in man. This has led to intensive research and strategic development of new vaccines around the world. Although less covered by the media after the appearance of the swine origin influenza A (H1N1), the H5N1 subtype with its high mortality rate ($\geq$ 60 %)[26] continues to cause sporadic human infection and still poses a pandemic threat.

In a pandemic scenario, the ideal vaccine should be easy to administer (needle-free), be inexpensive and provide effective protection by inducing both systemic and mucosal immune responses. For this reason intranasally administered vaccines are an attractive approach, especially in developing countries where health services are already overburdened. A needle free administration would eliminate the risk of HIV transmission in poor countries and have a greater public acceptance, thus being suitable for a global mass vaccination program.

Inactivated influenza vaccines are available in three formulations whole, split and subunit. Whole inactivated virus (WIV) vaccine is more immunogenic than split and subunit (SU) vaccines in unprimed individuals[66-68] as well as in naïve mice[69, 70]. Unfortunately, it was associated with higher reactogenicity, especially in children[67,71] and because of this most influenza vaccines used today are split or subunit vaccines. However, it has been suggested that the systemic reactions to seasonal whole virus vaccines may be due to egg-impurities in the influenza B strain formulated in the vaccine[72]. Two recent phase I clinical trials using current technology to produce a WIV vaccine (H5N1 and H2N2) demonstrated only mild reactogenicity and the vaccine was well tolerated by the participants[65, 68].

In man, vaccines containing novel avian subtypes have generally been poorly immunogenic[36, 73-75], using up to 90 µg HA or adjuvant and two doses are needed to induce satisfying antibody levels. The pandemic threat has lead to a re-evaluation of the WIV formulation because of its superior immunogenicity. An H5N1 WIV vaccine was recently tested in a clinical trial with or without aluminium adjuvant and was found to induce higher antibody levels in the non-adjuvanted groups[65].

Furthermore, a recent murine study concluded that the superior quality of the immune response after whole virus formulation could be explained by toll-like receptor signalling, especially stimulation of TLR-7, which resulted in a greater magnitude and a Th-1 skewed immune response. Efficient TLR-7 signalling seems to be lost in spilt and SU vaccines which contain much less viral ssRNA[76] or lacking the spatial organization of whole virion structure[70].

Previous work carried out by our group demonstrated that mice immunized IM with one dos of seasonal WIV (H3N2) vaccine displayed high serum IgG2a antibody levels indicative of a Th-1 biased profile, but after the second dose a more mixed Th-1/Th-2 profile was induced[69]. In contrast, further work with an avian WIV (H7N1) vaccine resulted in much lower levels of circulating antibodies, but similarly, a dominant Th-1 profile after one dose and a mixed profile after the second dose[77]. Bearing in mind these results, it was interesting to compare the IN administration route which is more similar to natural route of infection, against the standard IM administration route using another avian subtype, H5N1. We measured the kinetics and the magnitude of the humoral immune response in mice immunized (IM or IN) with two doses of 7.5 μg WIV vaccine (H5N1). This dose has in a study been found to be immunogenic in a study in man[65]. We further analyzed the T-helper profiles and the cytokine profiles. We found that both IM and IN immunization induced high levels of circulating influenza specific antibodies, where two doses of IM vaccine resulted in a mixed Th-1/Th-2 profile and significant concentrations of Th-2 cytokines. In contrast, the IN vaccine had an overall Th-2 biased profile, high concentrations of IL-17 and Th-1 cytokines. In addition, this route also induced a local IgA response after two vaccine doses.

5.1 The humoral immune response after intramuscular or intranasal vaccination

5.1.1 Both intramuscular and intranasal vaccination effectively induced haemagglutination inhibition antibodies in serum

Antibodies provide immediate protection and are the only mechanisms of the adaptive immune response that can block an infection[78]. Thus, eliciting high levels of serum antibody is one of the main goals of vaccination. The Committee for Medicinal Products

for Human Use (CHMP) has defined criteria that must be met annually for seasonal influenza vaccines to obtain a licence in Europe. One of these criteria is that HI titres should be ≥40 [79], which indicate 50% protection levels of serum anti-HA against infection[80]. However, this criterion may not be appropriate for avian viruses with a pandemic potential, indicating the need for more research into correlates of protection.

We found no or very low HI titres in both groups after one vaccination, compared to much higher titres previously reported with H3N2 WIV[69] or split vaccines in mice[81]. A rapid increase in titres was observed after the second vaccine dose and both groups had high HI titres. Although the HI titres were lower than those observed with seasonal vaccine, we found similar kinetics and magnitude compared to other avian WIV vaccine studies[77, 82]. The HI titres were also much higher than aluminium adjuvanted split H7N1 virus vaccine using high amounts of antigen (20-24 μg HA), in both mice and man[83, 84].

We found no significant differences in the HI titres between the two administration routes, although the IM group had two mice with HI titres after the first dose, which may be due to individual differences between the mice. The secondary response in the two groups was rapid and importantly the levels of HI antibody had only slightly declined twelve weeks post vaccination, indicating that both routes induced long-term immunity. Two doses of vaccine elicited HI titres considered to be protective and confirmed other findings that pandemic candidate vaccines of avian origin need two doses to reach the HI levels associated with protection (reviewed in[85])**.** However, protective efficacy may not always be directly correlated with the level of circulating antibodies, as shown in challenge experiments in animal models. Despite inducing low HI titres after vaccination, a number of vaccines still induced a significant protection from disease and death in mice and ferrets[38, 83, 86, 87]. Thus, we need a better understanding of the correlates of protection to avian viruses to assess vaccine-induced immunity. It might also be valuable to study T-cell responses more thoroughly. Moreover, serological assays including HI, have suffered from variability between different laboratories, the lack of standardization and use of blood cells from different species, thus making comparisons between studies difficult[88]. At present, a human H5N1 plasma standard has been prepared by the National Institute for Biological Standards and Control (NIBSC) and the inclusion of this in assays will allow improved comparison between vaccine trials[89].

5.1.2 Intramuscular vaccination induces high serum IgG antibody responses

Parenteral injection typically induces primarily HA-specific IgG (reviewed in[90]), which dominated the response in the IM group in this study. Serum IgG antibody is highly specific and very effective in neutralizing homologous virus strains (reviewed in[47]). The kinetics of the serum antibody response of the two groups during this study was similar. After the first dose, the primary response for the IM vaccinated animals was more rapid and stronger than in the IN group. However, after two doses of vaccine both vaccine groups elicited high concentrations of IgG, although significantly higher levels were observed in the IM group throughout the whole study. The IgG levels remained high 12 weeks after vaccination similarly to the HI titres, suggesting that both vaccine routes induced long lasting immunity. The induction of long lasting antibodies is interesting because previous pandemics appeared as waves of infection some time after the first outbreak[91].

Serum IgG is an important mediator in protection against influenza, and can transudate over the alveolar walls in the lungs[61], although it can also leak to the mucosal surfaces in the upper respiratory tract. However, in studies in mice infected with seasonal influenza H1N1, serum IgG titres correlates with virus titres in lung washes, but not in the upper respiratory tract[42, 92], thus indicating a more important role of immunity in the lower respiratory tract. Additionally, high IgG levels have been shown to limit viral shedding after infection of mice[93]. It has been reported by others that a concentration of 38 μg/mL serum IgG in aged mice resulted in undetectable viral titres of seasonal H1N1 in lung washes[94] after challenge with live H1N1 virus. It is therefore essential that vaccination induces IgG which neutralize virus in the lower respiratory tract thus preventing more serious complications of infection like influenza pneumonia. Therefore, our results suggest that the both groups would have cleared an infection and that the mice would have been protected against illness and pneumonia.

5.1.3 Antibody secreting cells in the spleen and bone marrow

The initial humoral response occurs in the spleen, nasal associated lymphoid tissue (NALT) and lymph nodes, but at a later stage of the immune response about 2-3 weeks

after a viral infection long-lived antibody secreting cells (ASC) home to the bone marrow, where they secrete antibodies over a long period[57]. We generally observed differences in magnitude between the spleen and bone marrow in both groups, especially after the second dose. Similarly to the serum IgG concentrations, intramuscular vaccination induced significantly higher numbers of IgG ASC than the IN group in the spleen after one vaccination and by day four after the second vaccination. However, by three weeks after the second dose, the numbers were similar. High numbers of influenza specific IgM ASC were also detected in the IM group, and later in the IN group. The IgM ASC cells we detected in the control mice most likely belonged to the so-called B1 lineage cells which express natural non-specific antibodies[95]. In the bone marrow one dose of vaccine induced only low numbers of ASC in both groups, indicative of a poor primary B-cell stimulation. However, much higher numbers were detected directly (four days) and three weeks after the second dose, suggesting that an additional dose is needed for better priming.

5.1.4 Intranasal vaccination induces significantly higher serum IgA concentrations than intramuscular vaccination

In addition to IgG, vaccination also induced anti-HA IgA. Although IgA is associated with mucosal surfaces, it functions as a second line of defence in serum by eliminating pathogens that have breached through the mucosa[96]. IgA is not normally induced after IM vaccination[42] and was not detected after one dose of IM vaccine. In the IN group, on the other hand, we detected serum IgA two weeks after the first vaccination. The secondary response in this group was more rapid and of a greater magnitude than the IM group. Serum IgA in the IM group was detected after the second vaccine dose, although it was only in low concentrations. Not surprisingly, the IN group had significantly higher numbers of IgA ASC in the spleen at all sampling points, indicative of a mucosal response and that the administration route is essential for IgA production. Also in the bone marrow, IgA ASC were significantly higher in the IN group three weeks after the second vaccination.

5.1.5 Intranasal vaccination induced a local IgA response after two vaccine doses

Natural influenza virus infection induces secretory IgA (S-IgA) which is secreted over the mucosal surfaces in the respiratory tract by plasma cells within the lamina propria[64], and

protects the epithelial surface which is the first target of influenza infection. We measured influenza-specific S-IgA from nasal washings (although we did not analyze for the secretory component), and found concentrations of S-IgA at or just above the detection limit after one dose of vaccine in both groups. After the second dose, there was a rapid increase in NW IgA in the IN group, which agrees with other studies of non-adjuvanted WIV vaccine[70].

In a murine study, it was found that a concentration higher than 15 ng/mL HA-specific IgA in nasal washes significantly correlated with reduced viral titres in nasal washes after influenza infection (H1N1)[94]. On the other hand, IgA in lung washes in the same study did not correlate with reduction in virus titres, confirming that S-IgA is secreted sparsely in the lower respiratory tract. However, these findings are dependent on the virus and if it produces a lower or an upper respiratory tract infection. If a concentration of 15 ng/mL can be applied to our results, the IN group would mainly have been protected from infection after two doses of vaccine. Nevertheless, a rapid decrease in IgA concentrations was observed after the peak magnitude of the response measured two weeks post second dose. Despite the fact that S-IgA was still measured at twelve weeks, the concentration had decreased by 6-fold, making it difficult to draw a conclusion as to how long the protection would have lasted. Furthermore, it might also be difficult to evaluate the protective effect of S-IgA alone against infection after vaccination because circulating immunity (serum IgG, IgA and IgM) is induced simultaneously and protection is multifaceted.

Secretory IgA is remarkably stable and exhibits prolonged function in secretions, and in contrast to serum IgG, S-IgA has the unique property that it can recognize antigenically drifted strains and thereby induce heterosubtypic immunity (reviewed in[47]). It has been reported that in mice immunized intranasally with H3N2, 50% survived a H5N1 challenge[97]. Therefore, the induction of S-IgA after vaccination is desirable. A noteworthy challenge with the H5N1 subtype is that the viruses have become phylogenetically different and are currently divided into 10 distinctive clades based on antigenic differences in the HA gene, and further into subclades[98]. Studies have shown that IN vaccines induce HI serum antibodies against both homologous and heterologous strains[99, 100], findings that make intranasal vaccination very attractive.

Furthermore, most split and SU vaccines delivered intranasally are poorly immunogenic and requires an effective adjuvant (reviewed in [63]). No mucosal adjuvants are currently approved for human use, the only inactivated IN vaccine formerly licensed was withdrawn from the market due to that the adjuvant *E. coli* heat-labile toxin was associated with cases of Bell's Palsy (facial paralysis)[40, 101]. Thus there is a need for finding a safe and effective mucosal adjuvant and it has been reported that delivering the vaccine sublingually may reduce the risk of vaccine-induced paralysis[102]. However, in our study, we found that non-adjuvanted WIV elicited a satisfactory mucosal response. Moreover, it would have been interesting to study the effect of lowering the antigen dose together with and without an effective adjuvant, given that dose sparing regimes are an important part of pandemic planning due to the limited capacity of the vaccine manufacturers.

5.2 Cytokine responses and IgG subclasses

There are no regulatory requirements for influenza vaccines to induce a cellular response, although cytotoxic T-lymphocytes are crucial in clearing viruses in the initial phase of infection (review in [48]), and in the recovery of mice with influenza pneumonia[50]. Experimental infection results in a strong type 1 response (IgG2a and IFN-γ)[93,103] and activation cytotoxic T-cells. In contrast, a type 2 response is associated with effective neutralization of virus[59]. In our study, intramuscular immunization elicited a strong IgG2a dominance (Th-1 profile) after one dose of vaccine, but we observed a more mixed Th1/Th-2 profile after the second dose. Similar results have been described in previous reports after WIV H7N1 and H5N1 vaccination[77,76] and also after seasonal whole virus vaccines[69, 70]. The high level of IgG2a antibodies in the IM group suggest that intramuscular vaccination might be more potent in inducing antibody-dependent cytotoxicity (ADCC).

Interestingly, despite being more similar to the route of natural infection, intranasal immunization elicited an IgG1 dominance (Th-2 profile) in both primary and secondary responses after vaccination. A Th-2 response is normally induced after subunit and split virus vaccine and can also be induced by adding aluminium adjuvant to the WIV vaccine, as reported by others[76, 104, 105]. Furthermore, a study conducted with intranasally administered WIV H1N1 resulted in a Th-1 response[70]. However, the latter used a different mouse model, and might not be comparable to our study. Moreover, we used a different HA subtype in our study.

The investigation of the cytokine profile was more complicated to interpret. As IL-4 and IL-5 are considered Th-2 markers, we would have expected that the IN group would have secreted higher amounts of these cytokines, as IL-4 is known to stimulate IgG1 production. Instead, we observed that the IM group secreted significantly higher levels of these cytokines. The IM group also secreted IL-10 and IFN-γ, confirming a mixed Th-1/Th-2 profile as observed with the IgG subclass distribution, thus enhancing both cell mediated and humoral responses. Moreover, there was a higher production of IL-10 (which is considered a Th-2 cytokine) in the IN group. IL-10 has been found to induce class switching of antigen-specific B-cells to become IgA-committed plasma blasts (reviewed in[64]), thus enhancing the secretion of IgA and which was reflected by the high levels of serum and local IgA in this group.

Although the IN group was characterized by a Th-2 profile according to the dominance of IgG1, we also found high concentrations of IFN-γ, indicating that there was potentially a cytotoxic response as well. Moreover, also the Th-1 cytokine IL-2 was secreted by this group. A major advantage of a cytotoxic response is that CTL are effective in clearing drifted viruses, their targets are not the surface proteins, but particularly the nucleoproteins and matrix proteins which are conserved between the influenza A strains[106]. Experimental H5N1 infection of mice also induced elevated levels of IFN-γ and IL-10[107], and might suggest the response observed after IN vaccination is more similar to natural infection. IFN-γ has been shown to up-regulate the expression of the poly immunoglobulin receptor (pIgR) on epithelial cells[108], thus enhancing secretion of locally produced S-IgA. Therefore, it would have been interesting to evaluate the cytokine response in the NALT or local lymph nodes after IN immunization, to assess if IFN-γ also was secreted at these immunological sites.

It is noteworthy that a high concentration of IL-17 was secreted in the group vaccinated IN, whilst no IL-17 was detectable in the IM group. IL-17 is produced upon inflammation, and the high level can be explained by intranasal vaccination inducing a local inflammatory reaction. A Th-17 profile is advantageous because IL-17 stimulates recruitment of neutrophils which enhances phagocytosis of antigens. In addition, $CD4^+$ cells secreting IL-17 have been detected in the lung after influenza infection[109], thus enhancing protection against influenza by the influx of neutrophils.

5.3 Conclusion

In this study, we have demonstrated that there are the substantial differences in the immunological profiles after intramuscular and intranasal vaccination. An H5N1 inactivated whole virus vaccine containing 7.5 μg HA induced a good systemic humoral immune response in both experimental groups after two doses, which would have fulfilled the CHMP criteria of an HI titre ≥40 for a seasonal vaccine in man. The IM group had a predominant Th-1 profile after one dose, but shifted to a more mixed Th-1/Th-2 profile after two doses, thus promoting a humoral response and potentially a cytotoxic response. On the other hand, the IN group displayed an overall Th-2 profile, but also secreted Th-1 cytokines. In addition we measured high concentrations of IL-17 in this group, which stimulates phagocytosis by neutrophils. Finally, the IN group produced significant levels of S-IgA, which is crucial in preventing viral shedding in the upper respiratory tract.

Both IgG and IgA antibodies are important in defence against influenza infection and we therefore suggest that the IN vaccine would be the best alternative for further research, as local IgA would provide the first line of defence and IgG would serve as a back-up if IgA should fail to prevent the infection. There is an urgent need to develop immunization strategies that will optimize protection in naive individuals, and a non-invasive vaccine would also be suitable in developing countries. Although, for a human application it might be easier to licence an intramuscular vaccine, since this is the conventional administration route. Regardless of this we suggest that the intranasal WIV vaccine should be further investigated as a pandemic candidate vaccine against influenza H5N1.

Avian virus vaccines are poorly immunogenic, and here we have also demonstrated that the use of whole-virus vaccine can be dose-sparing, when comparing other studies using 90 μg HA of split virus vaccine. Dose sparing is very important as the global vaccine supply will be limited in a pandemic[110]. Finally, all these vaccine studies conducted to date both in animal models and human clinical trials have allowed the world to be better prepared to effectively meet the current swine origin influenza A (H1N1) pandemic.

5.4 Further studies

In our study we have evaluated the humoral and cytokine response to H5N1 WIV in a murine model. There are many further studies which could be conducted to evaluate this promising vaccine to evaluate in more detail the immune response. It would have been interesting to test the serum samples in an HI assay using different clades of viruses to see if the vaccine induced cross-reactive antibodies between strains, and also conduct a virus neutralising assay to homologous and heterologous strains.

Furthermore, we could have tested the quality of the T cell response by studying multifunctional $CD4^+$ T cells (secreting for example IFN-γ, IL-2 and TNF) from spleen, NALT and lymph nodes using flow cytometry. Vaccination induced Th-1 cytokines and therefore a $CD8^+$ cytotoxicity assay could have been conducted to evaluate if there was a cytotoxic response.

Dose sparing is an important part of pandemic planning and more work on testing different doses of haemagglutinin should be conducted, first of all lower doses. Also the use of good mucosal adjuvant could further have reduced the antigen dose for IN vaccination and enhanced the immune response. Next, the formulations should be evaluated in a lethal murine challenge model with homologous and heterologous strains to evaluate the protective efficacy at different doses. The vaccine should be further tested in other animal models, particularly the ferret, to confirm our findings in the murine model.

We also suggest detailed evaluation of the immune response by evaluating the memory B and T-cell responses, as well as studying the long lasting local and systemic response after six and 12 months.

The world is currently in the early stages of a pandemic and with our promising results using WIV, we would also suggest that evaluation of a pandemic H1N1 WIV vaccine is an important next step.

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